THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME XXXIX OCTOBER, 1961 NUMBER 4

IMMEDIATE AND DELAYED NEPHROTOXIC NEPHRITIS IN RATS

THE ROLE OF COMPLEMENT FIXATION

ARNOLD VOGT, M.D.,* AND HANS G. KOCHEM, M.D.

From the Institute of Pathology, University of Tübingen, Tübingen, West Germany

Antikidney serum from the duck (DAKS) injected into rats usually initiates nephritis after a lag period of 3 to 20 days.[1-3] On the other hand, the injection of rabbit antikidney serum (RAKS) causes renal damage almost immediately.[4-6] The latter may be detected by the appearance of proteinuria within hours after the injection.

Antikidney serum can be demonstrated by the appearance of isotopes or fluorescein-tagged antiserums in the kidneys following their intravenous injection.[7-9] Both nephrotoxins, DAKS and RAKS, are bound to the kidney immediately; the difference in their mode of action must be influenced by other factors. Pressman, Korngold and Heymann[10] indicated that for the development of a nephrotoxic nephritis, complement fixation might be necessary. They extended Kay's hypothesis[2,3] that a "silent phase I" takes place by fixation of the duck antikidney serum without fixation of complement. Cell damage begins only after the fixation of complement, because a lysis of erythrocytes and bacteria by antibodies takes place only in the presence of this substance. Pressman and co-workers based their contention that the duck antibodies lacked the ability to bind complement *in vitro* on the observations of Izumi[11]; antibodies from the rabbit, however, appeared to possess this quality. In recent years, several attempts have been made to clarify the role of complement in experimental nephritis. The estimation of the level of

Accepted for publication, May 11, 1961.

* Present address: Hygiene Institut der Universitaet Freiburg, Germany.

complement in the blood of experimental animals permitted only tentative conclusions as to its fixation in the kidney.[12-14]

In the present study, complement fixation in the renal glomeruli in the course of experimental nephrotoxic nephritis was demonstrated histoserologically. The experiments were made on unfixed kidney sections, utilizing the method of Klein and Burkholder.[15,16] Complement fixation was detected in the glomerular tufts immediately after the injection of RAKS. If DAKS was injected, complement fixation could be demonstrated only after a lag period of at least 3 days.

Material and Methods

Preparation of Antikidney Serums

Under ether anesthesia, the kidneys of white inbred rats were cleansed by the infusion of 0.9 per cent saline introduced via the abdominal aorta, and renal tissue was then homogenized in a Star mixer. The kidney emulsion was thereafter injected twice a week, 16 times in all, into 5 rabbits and 5 ducks. Each animal received an inoculum consisting of the total amount of emulsion from 48 rat kidneys. Ten days after the last injection, blood was drawn from rabbits and ducks under ether anesthesia. The serums were inactivated in a water bath (30 minutes at 56° C.) and filtered through a Seitz filter. Pooled serums were kept as rabbit anti-rat kidney serum (RAKS) and duck anti-rat kidney serum (DAKS) at −20° C. until used.

Preparation of Fluorescein-Tagged Antibodies

Rabbit Anti-Duck Globulin (RADG). Gamma globulin was procured from the duck by precipitation of the serum with half-saturated ammonium sulfate. It was injected into rabbits, partly with Freund's adjuvant.

Duck Anti-Rabbit Globulin (DARG). As in the rabbit, antibody was prepared by injection of rabbit gamma globulin into ducks.

Anti-Complement. The preparation of anti-complement has been described thoroughly by Klein and Burkholder.[15,16]

All antibodies were isolated by ammonium sulfate precipitation and tagged with fluorescein isothiocyanate following the directions of Coons and Kaplan.[17] The tagged serums were absorbed with equal amounts of dried pig liver powder. Thereafter, the antibodies were passed through a Seitz filter. Merthiolate (1:10,000) was added, and the material stored at −20° C.

Guinea pig complement was procured freshly by heart puncture; the blood was allowed to clot at 4° C. and centrifuged. Complement was used in a 1 to 20 dilution with buffered saline; merthiolate was not added. To chelate the complement, 0.2 M ethylenediamine tetra-acetic acid (EDTA) was added to a final dilution of 1 to 20. Inactivation was accomplished by heating the serum in a water bath at 56° C. for 30 minutes.

Demonstration of Antikidney Serums

In order to demonstrate the antikidney serums, sections were cut at 5 μ in a cryostat and covered with 1 to 2 drops of fluorescein-tagged antibody: (a) anti-rabbit gamma globulin from the duck (DARG); and (b) anti-duck gamma globulin from the rabbit (RADG). (In a number of cases untagged RADG was first added, and then, after rinsing the section in buffered saline, fluorescein-marked DARG was applied.) The sections were incubated in a wet Petri dish at 37° C., rinsed with buffered saline, and covered with 0.9 per cent saline.

Demonstration of Complement Fixation

Sections were covered with 1 to 2 drops of fresh guinea pig complement in a dilution of 1 to 20 and incubated in a wet Petri dish at 37° C. for 30 minutes. They were then rinsed in buffered saline (pH 7.2) for 10 minutes, covered with 2 drops of fluorescein-marked anti-complement and incubated again for 15 to 30 minutes under identical conditions. After another rinsing in buffered saline, the sections were ready for examination.

Controls

The following control scheme was used in each instance: (1) Two sections of kidney with Masugi nephritis induced in the rat by RAKS served as models. Before the tagged anti-complement was added, one of these was covered with complement, the other with inactivated complement. (2) One section was examined unstained to determine its autofluorescence. (3) One section of the rat kidney was covered with tagged anti-complement in order to detect or exclude nonspecific absorption of tagged anti-complement in the tufts. (4) Before being covered with fluorescein-tagged anti-complement, one section was first treated with complement inactivated by heat; and (5) one section with complement was chelated with EDTA, in order to exclude nonspecific absorption of complement unrelated to antigen-antibody complexes. (6) For the elimination of anti-complementary factors in duck antiserums, sections that showed a complement fixation with the method described above were utilized. In order to avoid anti-complementary factors in duck antikidney serum, sections were covered with (a) duck antikidney serum or (b) normal duck serum, and efforts were made to demonstrate complement fixation. Furthermore, complement was first added, then DAKS, and finally, fluorescein-tagged anti-complement.

Conventional Histologic Examination

Samples of kidney fixed in formalin were embedded in paraffin; sections were cut at 3 to 5 μ and stained with hematoxylin and eosin (H and E) and by the periodic acid-Schiff (PAS) method.

Fluorescence Microscopy and Photography

A Leitz dialux microscope with a high pressure mercury bulb CS 150 W. was utilized; filters consisted of a cuvette with 1 per cent $CuSO_4$; a UV_1 filter; a UG_1 2 mm. filter; and Euphos UVa filters, 2.5 mm. Photographs were made with 17° DIN films; exposure times were between 10 and 20 minutes. Control sections were exposed for the same period.

Experimental Procedures

Group I consisted of 6 control rats. Under ether anesthesia, 3 of these received 2 ml. of normal inactivated duck serum. All injections were given into the tail veins. There were no clinical symptoms in this group.

Group II included 21 white inbred rats which received RAKS in single intravenous doses. The amount of RAKS injected ranged from 0.125 ml. to 2 ml. Ten rats received between 0.125 ml. and 0.4 ml.; 10 rats were given between 0.5 ml. and 1 ml., and 1 rat received 2 ml.

Group III consisted of 25 rats which received between 1 ml. and 8 ml. of DAKS intravenously. Fifteen rats were given 1 ml.; 8 rats received 4 ml., and 2 rats received 8 ml. as total dosage. Up to 4 ml. were given in a single dose; larger amounts were given when injected on 2 consecutive days.

With dosages up to 0.5 ml. of RAKS and 4 ml. of DAKS, rats showed no severe manifestations. With larger doses, a number of rats died of pulmonary edema immediately or within 15 minutes. These animals were excluded from the results.

The left kidney of each rat was removed under ether anesthesia within 5 days

after the injection of a nephrotoxic antibody or the foreign serums. The presence of the antibodies and complement fixation was determined by the procedures described above. The right kidney was removed and examined up to 8 weeks after serum injection.

Results

CLINICAL OBSERVATIONS

Clinical evidence of illness was determined by quantitative estimation of proteinuria. Since healthy animals excrete small amounts of protein in the urine, only a proteinuria above 1 mg. per ml. was considered significant. A large number of estimations prior to the experiments showed proteinuria among normal rats up to 0.3 mg. per ml. (Esbach's method). The animals in Group I (controls) exhibited no proteinuria after the injection of normal rabbit and duck serum.

Group II. The introduction of as much as 0.4 ml. RAKS usually caused no proteinuria even after a lag period. Only dosages of 0.5 ml. or more were followed by proteinuria in a considerable number of animals; between 2 and 8 mg. per ml. appeared within hours after injection. Here, too, an all-or-none response was observed. Either the rat became sick at once (without a lag period) or not at all. In animals receiving relatively small amounts of RAKS, proteinuria ended after 2 or 3 weeks, and they were thus considered clinically healthy at this time. After a dosage of 0.8 ml. of RAKS or more, rats continued to exhibit proteinuria even after 4 to 5 weeks.

Group III. None of the rats receiving DAKS exhibited proteinuria during the first days after injection, even after the administration of 8 ml. Proteinuria appeared as late as the sixth day after injection of 4 ml. of DAKS (1 to 7 mg. per ml.). Once the protein excretion had begun, it continued almost without exception until the end of the experiment.

HISTOLOGIC FEATURES

The histologic findings are recorded only briefly, since the main task in this study was to determine complement fixation in nephrotoxic nephritis. Rats clinically ill showed the characteristic swelling of the glomeruli and glomerular tufts previously described by Masugi[4] (Figs. 1 and 2). Neutrophils and eosinophils in the tufts were increased in number. A slight increase in glomerular epithelial content and a swelling of these cells were observed in the lag period among the animals receiving DAKS. In later stages a thickening of the glomerular basement membrane was noted. Animals with marked proteinuria showed hyaline droplet degeneration in the distal and proximal convoluted tubules. The rats that appeared clinically healthy exhibited only minor alterations not easily distinguishable from normal structures; in later stages there were no glomerular lesions in these animals.

FLUORESENCE STAINING

Freshly prepared, untreated sections of kidney showed no significant autofluorescence; control sections exposed to fluorescein-coupled anti-complement disclosed only a slight, diffuse green fluorescence. In both instances the glomeruli were free of fluorescence and appeared as empty dark areas. Similar findings were obtained with the control sections treated with inactivated complement and those exposed to fluorescein-coupled anti-complement. The weak, nonspecific staining in these sections increased very little with longer exposure time; the glomeruli remained as dark, empty, more or less round spaces. In very few instances only were the glomeruli barely diffusely stained; this weak, nonspecific stain was much less intense than that in the surrounding tubules.

These were the findings after treatment of the sections with heat-inactivated and EDTA-chelated complement. When exposed to active complement and, thereafter, to fluorescein-marked anti-complement, a bright, distinct green fluorescence appeared in the glomerular tufts in cases with complement fixation, i.e., where the anti-kidney serum was deposited (Fig. 5). The histoserologic phenomenon was considered positive only if the basement membrane of the glomerular tuft showed a distinct, bright green fluorescence and if the nonspecific staining was insignificant by comparison. Obviously the corresponding controls with inactivated complement had to be negative. The demonstration of the antikidney serum was, compared with that of the complement fixation, more intense and restricted exclusively to the glomeruli, whereas the tubules were not stained.

Group I. Observations After Injection of Rabbit or Duck Serum

Normal rabbit serum could not be demonstrated in the kidneys of rats by means of fluorescein-coupled duck anti-rabbit globulin (DARG). The attempt to demonstrate normal duck serum by means of fluorescein-marked rabbit anti-duck globulin (RADG) was also unsuccessful. In this group of 6 animals, the left kidney was removed within 3 days of the injection, and the right kidney was examined after 3 to 4 weeks. No complement fixation was evident in any of the kidneys (Table II).

Groups II and III: Detection of Antikidney Serum with Fluorescent Antiserum

In group II RAKS was demonstrable in every kidney of the animals receiving injections. Exposure of the sections to fluorescein-coupled DARG caused a very strong and distinct staining of the glomerular tufts (Fig. 3). With lesser amounts of RAKS, there was slightly less intense fluorescence, but even after the injection of as little as 0.125 ml., a dis-

tinct green staining of tufts was evident. Fluorescence was reduced after several weeks, but even after 5 to 6 weeks, RAKS was still demonstrable.

As in group II, the animals receiving DAKS (group III) showed evidence of glomerular localization with all dosages utilized. Glomerular fluorescence was the same as in group II although the brilliance was

TABLE I

HISTOSEROLOGIC EVIDENCE OF COMPLEMENT FIXATION IN EXPERIMENTAL NEPHROTOXIC NEPHRITIS

Anti-rat kidney serum	Amt. injected (ml.)	Complement fixation	Number of rats											
			Days after injection *							Weeks after injection †				
			1	2	3	4	5	6	7	2	3	4	5	6
RAKS	0.125–0.4	Yes	6	2	1	1			1	3	0	0	0	
		No	0	0	0	0			1	1	1	2	1	
	0.5 – 2.0	Yes	6	2	1	1	1		1	4	2	2	1	
		No	0	0	0	0	0		0	0	0	0	0	
DAKS	1.0	Yes	0	0	(1)‡	(1)	(2)		2	5	3	2	1	0
		No	6	3	2	0	0		0	0	0	1	0	1
	2.0 – 8.0	Yes	0	0	0	(1)	2		1	2	1	1	2	1
		No	3§	2§	2	0	0		0	0	0	0	0	0

* Examination of left kidney.
† Examination of right kidney.
‡ Parentheses denote dubious complement fixation.
§ One animal died during removal of the left kidney.
RAKS = antikidney serum from rabbit. DAKS = antikidney serum from duck.

somewhat less intense (Fig. 6). Results were also identical with direct exposure of sections to RADG and with the "sandwich method": first covering the section with unmarked RADG and then with fluorescein-tagged DARG. The results with the latter method were somewhat less intense than those obtained by the direct detection of DAKS, which was not manifest until 6 weeks after injection.

Histoserologic Detection of Complement Fixation

Group II (RAKS). Complement fixation was observed in glomerular tufts within a few minutes after injection of RAKS. Localization was identical with that of RAKS (Fig. 4). Moreover, complement fixation occurred in cases which received only one quarter of the dose necessary to induce clinical manifestations of nephritis. Following the injection

of relatively small doses, complement fixation was no longer evident after 3 weeks; with larger doses leading to proteinuria, glomerular complement fixation continued even after 5 weeks. In several rats complement binding complexes were detected in a few distal or proximal convoluted tubules and in occasional intratubular protein casts.

Group III (DAKS). In this group of animals, complement fixation was inapparent during the first 3 days after the administration of DAKS. Even utilizing a dose of 8 ml. of DAKS (twice the amount that, after a lag period of 6 days, regularly caused proteinuria), the kidneys were incapable of fixing complement and were thus free of specific fluorescence. It was only after the third day that the complement fixation reaction became evident (Fig. 7). In several comparable experiments, complement fixation was manifest during the lag period before proteinuria appeared. Clinically healthy animals also showed a positive complement fixation from the third to sixth days after DAKS injection. Usually the complement fixation persisted for a longer period than in group II (Table I).

Anticomplementary Factors in DAKS. An attempt was made to determine whether the failure to demonstrate complement fixation during the first days after injection of DAKS was caused by anti-complementary factors in DAKS or duck serum. The results are shown in Table II. Neither duck serum nor DAKS can hinder or eliminate complement fixation, whether injected before or after application of RAKS (Fig. 8). In Table III it is shown that sections of animals in group III (rats whose kidneys bound complement in previous tests) treated with duck serum or DAKS, failed to exhibit a complement fixation reaction.

Discussion

Apparently our observations confirm the explanation for the lag period offered by Pressman and co-workers.[10] These authors contended that the complement fixation capacity of a specific nephrotoxic antibody was established decisively at the outset of the nephritis. Basing their opinion on Izumi's experiments,[11] they assumed that DAKS did not fix complement, whereas RAKS did. In fact, our investigation has shown that complement fixation appeared at the localization site of antikidney serum shortly after the injection of RAKS; however, in the case of DAKS, complement fixation appeared only after a lag period of 3 days. This delay in complement fixation was not attributable to an anti-complementary factor in DAKS or in duck serum. One must accept that the duck antibody does not consume complement after fixation at the basement membrane, i.e., the antigen.

It is necessary to remark at this point that our work was carried out

only with guinea pig complement. We assumed the probability that endogenous rat complement acted as did guinea pig complement. Histoserologic evidence of an endogenous complement fixation could not be determined for two reasons: (1) There are cross reactions between complement and globulins in the same serum; and (2) it is not possible to provide a control with inactive complement.[15] Despite the qualitative

TABLE II

THE EXCLUSION OF ANTI-COMPLEMENTARY FACTORS IN DAKS (CONTROL AND IN VITRO EXPERIMENTS)

Sequence of injection of serum	Fluorescein-tagged antiserum		Histoserologic evidence of complement fixation 24 hr. after injection
	Rabbit globulin	Duck globulin	
RS	oo	oo	oo
DS	oo	oo	oo
RAKS, DS	+	oo	+
RAKS, DAKS	+	+	+
DAKS	oo	+	oo
DAKS, RAKS	+	+	+

RS = rabbit serum. DS = duck serum.
RAKS = antikidney serum from the rabbit. DAKS = antikidney serum from the duck.
oo = negative 24 hours and 3 to 4 weeks after injection of serum.
+ = positive

TABLE III

THE EXCLUSION OF ANTI-COMPLEMENTARY FACTORS IN DAKS *

Sequence of exposure	Complement fixation
C, AC-F	+
Cch, AC-F	o
DS, C, AC-F	+
C, DS, AC-F	+
Cch, DS, AC-F	o
DAKS, C, AC-F	+
C, DAKS, AC-F	+
Cch, DAKS, AC-F	o

* Sections were procured from the kidneys of rats treated with RAKS.
C = guinea pig complement in a dilution of 1:20.
Cch = EDTA-chelated complement.
DS = duck serum.
DAKS = antikidney serum from the duck.
RAKS = antikidney serum from the rabbit.
AC-F = fluorescein-tagged anti-complement.

and quantitative differences in the complement systems of various species, there are great similarities. Thus, the complement components of many species are easily interchangeable.[18–21] It appeared to us that the failure of complement fixation had a causal connection with the lag period observed. In keeping with the opinions of Pressman and co-

workers,[10] there apparently was complement fixation in phase II (Kay[2,3]), accompanied by the formation of endogenous antibodies against the antikidney serum injected. However, the mechanism of the nephrotoxic nephritis seems to be more complicated than Pressman and co-workers had assumed, since an exact temporal coincidence between the onset of proteinuria and the positive complement fixation was not evident. Furthermore, complement fixation was always positive before the onset of proteinuria.

It was surprising that complement fixation was positive even after subliminal dosages of nephrotoxic serums and without clinical signs, i.e., proteinuria. In the present experiments complement fixation was demonstrated immediately after injection of RAKS, whereas it became manifest only following a lag period when subliminal dosages of DAKS were administered. Accepting the common opinion that additional participation of complement causes cell damage, we must conclude that proteinuria is an indication of considerable kidney damage and that less pronounced damage may remain clinically occult. On the other hand, complement fixation at the site of nephrotoxic serum localization appears to represent only an incidental indication of antigen-antibody reaction in renal glomeruli and presumably a process without pathogenic significance. In favor of this is the observation that DAKS causes an immediate proteinuria in a small percentage of rats.[22-24] The length of the lag period appears to depend on the strength or the amount of nephrotoxic serum introduced. The DAKS used by us was not sufficiently potent to cause a sudden onset of the nephritis without a lag period to indicate whether experimental nephritis may be induced without complement fixation. It is also probable that the duck, i.e., the donor of the nephrotoxic serum, forms two types of antibodies, one capable of binding complement after reaction with the corresponding antigen. A third possible explanation is that complement fixation may constitute an additional damaging factor in the course of the experimental nephritis.

In our opinion the fixation of the antikidney serum indicates prior cell damage which, depending upon its intensity, may lead to a clinically apparent disease. The simultaneous occurrence of complement fixation enhances the cell damage. That complement fixation is not the only factor causing injury is shown by the fact that the injection of RAKS in amounts too small to provoke clinical manifestations is associated with evidence of immediate complement fixation. Doerr[25] questioned the widely accepted view that only complement fixation causes cell damage, since, as shown by Robertson,[26] antiserums may destroy bacteria and protozoa in the absence of complement. The addition of complement causes an increase of the death rate, whereas a lysis of bacteria

and protozoa in the absence of complement may be demonstrated to only a small extent. We believe that complement in experimental nephrotoxic nephritis plays a similar role, i.e., it constitutes an additional damaging factor.

Summary

In the course of experimental nephritis, induced in the rat by antikidney serums from the rabbit and the duck, histoserologic evidence of complement fixation was demonstrated in the rat kidney. Of particular interest was the possible influence of complement fixation on the renal lesion.

If serum from normal rabbits or ducks was injected into rats, neither were demonstrable by fluorescein-tagged antibodies. Histoserologic evidence of complement fixation was also lacking.

Anti-rat-kidney rabbit serum in a dosage of 0.5 ml. caused immediate proteinuria in the rat. The antikidney serum injected was fixed to the basement membrane of the glomerulus immediately. At the site of localization of rabbit antikidney serum (RAKS), complement fixation was immediately demonstrable, and could be observed even if the amount of RAKS was insufficient to cause clinical evidence of nephritis. Complement fixation remained positive for 5 to 6 weeks after the introduction of RAKS.

The intravenous injection of duck antikidney serum (DAKS) caused proteinuria only after 6 to 9 days although tagged DAKS was demonstrable in glomerular tufts immediately after its introduction. Complement fixation was never positive before the third day but always appeared before the onset of proteinuria. It was found regularly after a lag period in rats which did not become ill clinically.

The importance of complement fixation at the site of antikidney serum localization in the course of experimental nephritis is discussed.

References

1. Masugi, M. Über die experimentelle Glomerulonephritis durch das spezifische Antinierenserum. Ein Beitrag zur Pathogenese der diffusen Glomerulonephritis. *Beitr. path. Anat.*, 1934, **92**, 429–466.
2. Kay, C. F. The mechanism by which experimental nephritis is produced in rabbits injected with nephrotoxic duck serum. *J. Exper. Med.*, 1940, **72**, 559–572.
3. Kay, C. F. The mechanism of a form of glomerulonephritis; nephrotoxic nephritis in rabbits. *Am. J. M. Sc.*, 1942, **204**, 483–490.
4. Masugi, M. Über das Wesen der spezifischen Veränderungen der Niere und der Leber durch das Nephrotoxin bzw. das Hepatotoxin. Zugleich ein Beitrag zur Pathogenese der Glomerulonephritis und der eklamptischen Lebererkrankung. *Beitr. path. Anat.*, 1933, **91**, 82–112.
5. Heymann, W., and Hackel, D. B. The early development of anatomic and

blood chemistry changes in the nephrotic syndrome in rats. *J. Lab. & Clin. Med.*, 1952, **39**, 429–436.

6. Lippman, R. W.; Marti, H. U., and Jacobs, E. E. Rapid effects upon the renal circulation produced by nephrotoxic globulin administration in the rat. *Proc. Nat. Acad. Sc.*, 1952, **38**, 260–263.
7. Pressman, D., and Keighley, G. The zone of activity of antibodies as determined by the use of radioactive tracers; zone of activity of nephrotoxic antikidney serum. *J. Immunol.*, 1948, **59**, 141–146.
8. Mellors, R. C.; Siegel, M., and Pressman, D. Analytical pathology: histochemical demonstration of antibody localization in tissues, with special reference to the antigenic components of kidney and lung. *Lab. Invest.*, 1955, **4**, 69–89.
9. Ortega, L. G., and Mellors, R. C. Analytical pathology. IV. The role of localized antibodies in the pathogenesis of nephrotoxic nephritis in the rat. *J. Exper. Med.*, 1956, **104**, 151–170.
10. Pressman, D.; Korngold, L., and Heymann, W. Localizing properties of anti-rat-kidney serum prepared in ducks. *Arch. Path.*, 1953, **55**, 347–348.
11. Izumi, F. Experimental studies on glomerulonephritis. I. Serological studies about duck immuno-serum against rabbit's kidney, serum, and blood cells (nephrotoxic immuno-serum, haemolysins, and precipitins). *Folia endocrinol.*, 1940, **16**, 532–602.
12. Stavitsky, A. B.; Hackel, D. B., and Heymann, W. Reduction of serum complement following *in vivo* tissue antigen-antibody reactions. *Proc. Soc. Exper. Biol. & Med.*, 1954, **85**, 593–596.
13. Lange, K., and Wenk, E. J. Investigations into the site of complement loss in experimental glomerulonephritis. *Am. J. M. Sc.*, 1954, **228**, 454–460.
14. Pfeiffer, E. F.; Schöffling, K.; Bruch, H. E., and Spielmann, W. Masugi-Nephritis und Serumkomplement der Ratte. (Speziesgebundene Unterschiede im pathogenetischen Mechanismus der Masugi-Nephritis von Ratte und Kaninchen). *Ztschr. ges. exper. Med.*, 1954, **122**, 446–464.
15. Klein, P., and Burkholder, P. Ein Verfahren zur fluoreszenzoptischen Darstellung der Komplementbindung und seine Anwendung zur histo-immunologischen Untersuchung der experimentellen Nierenanaphylaxie. *Deutsch. med. Wchnschr.*, 1959, **84**, 2001–2004.
16. Klein, P. G., and Burkholder, P. M. Studies on the antigenic properties of complement. II. Analysis of specific agglutinins against certain components of guinea pig complement fixed on sensitized sheep erythrocytes. *J. Exper. Med.*, 1960, **111**, 107–117.
17. Coons, A. H., and Kaplan, M. H. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. *J. Exper. Med.*, 1950, **91**, 1–13.
18. Hegedüs, A., and Greiner, H. Quantitative Bestimmung der Komplementbestandteile. *Ztschr. Immunitätsforsch.*, 1938, **92**, 1–9.
19. Cushing, J. E., Jr. A comparative study of complement. II. The interaction of components of different species. *J. Immunol.*, 1945, **50**, 75–89.
20. Vorlaender, K. O. Das Komplement in Grosstierseren. 3. Die Bindung hämalysierender Serumfraktionen an einen Antigen-Antikörperkomplex. *Ztschr. Immunitätsforsch.*, 1950, **107**, 302–309.
21. Rice, C. E., and Crowson, C. N. The interchangeability of the complement components of different animal species; in the hemolysis of sheep erythrocytes sensitized with rabbit amboceptor. *J. Immunol.*, 1950, **65**, 201–210.

22. STAVITSKY, A. B.; HEYMANN, W., and HACKEL, D. B. Relation of complement fixation to renal disease in rats injected with duck antikidney serum. *J. Lab. & Clin. Med.*, 1956, **47**, 349–356.

23. EHRICH, W. E.; FORMAN, C. W., and SEIFER, J. Diffuse glomerular nephritis and lipid nephrosis. Correlation of clinical, morphological, and experimental observations. *Arch. Path.*, 1952, **54**, 463–503.

24. HASSON, M. W.; BEVANS, M., and SEEGAL, B. C. Immediate or delayed nephritis in rats produced by duck anti-rat-kidney sera. *Arch. Path.*, 1957, **64**, 192–204.

25. DOERR, R. Das Komplement. Springer Verlag, Wien, 1947, 74 pp.

26. ROBERTSON, M. An *in vitro* study of the action of immune bodies called forth in the blood of rabbits by injection of the flagellate protozoon *Bodo caudatus*. *J. Path. & Bact.*, 1934, **38**, 363–390.

Reagents were supplied by Dr. Paul Klein, *Institut für Hygiene und Mikrobiologie* of the Medical Academy, Düsseldorf, Germany.

The serums were prepared with financial assistance from the *Deutsche Forschungsgemeinschaft* and the *Freunde und Förderer der Medizinischen Akademie*, Düsseldorf, Germany.

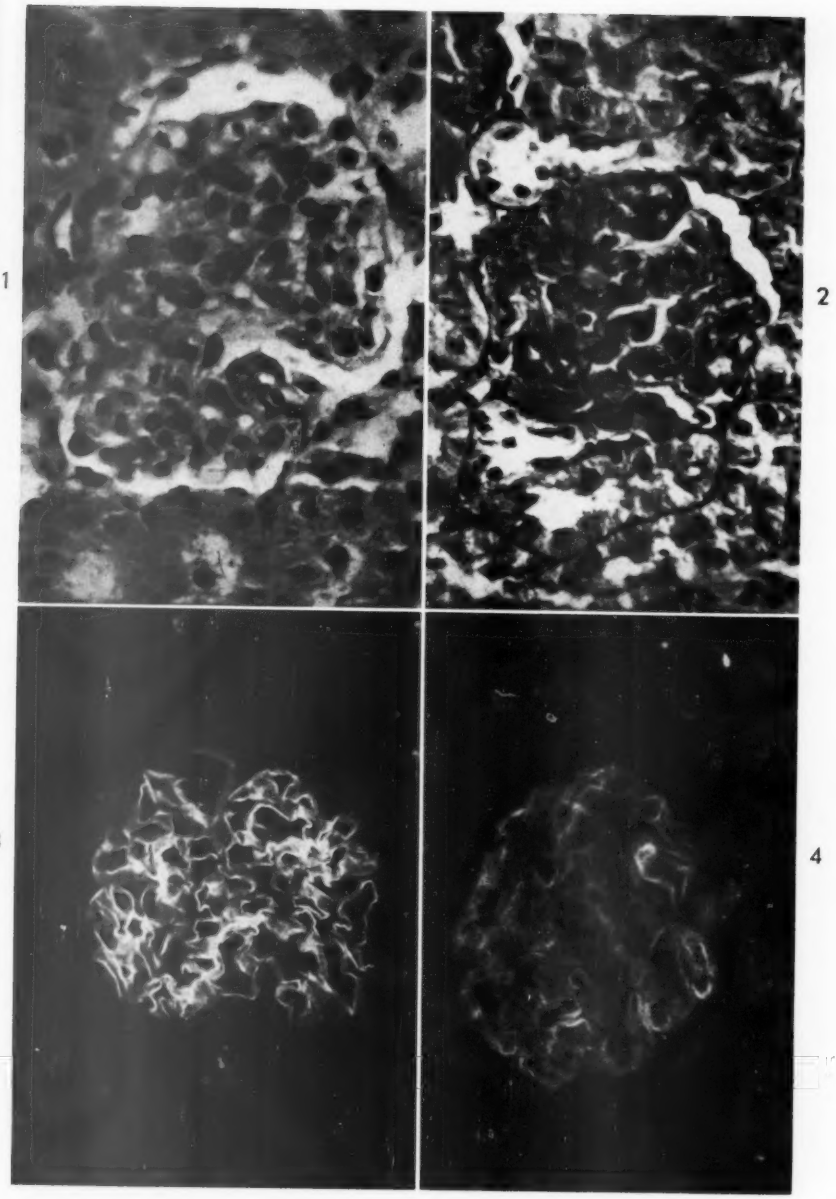
LEGENDS FOR FIGURES

FIG. 1. RAKS (1 ml.) injected 4 weeks earlier. There is swelling of glomerular tufts and an increase in cellularity. Basement membrane is thickened. Periodic acid-Schiff (PAS) stain. × 450.

FIG. 2. DAKS (4 ml.) injected 4 weeks earlier. There is membranous thickening and localized hyalinization of glomerular tufts. Adhesions appear between the tufts and the capsule. PAS stain. × 450.

FIG. 3. RAKS injection, 5 hours earlier. Section is stained with duck anti-rabbit globulin. The injected RAKS is localized in the basement membrane of the glomerular tufts. × 450.

FIG. 4. RAKS injection (0.5 ml.), one hour earlier. Section is stained with fluorescein-coupled anti-complement after treatment of the unfixed section with guinea pig complement. As in Figure 5, specific fluorescence is found in the basement membrane. This indicates that at the site of RAKS deposition, complement fixation can be found immediately after injection. × 450.



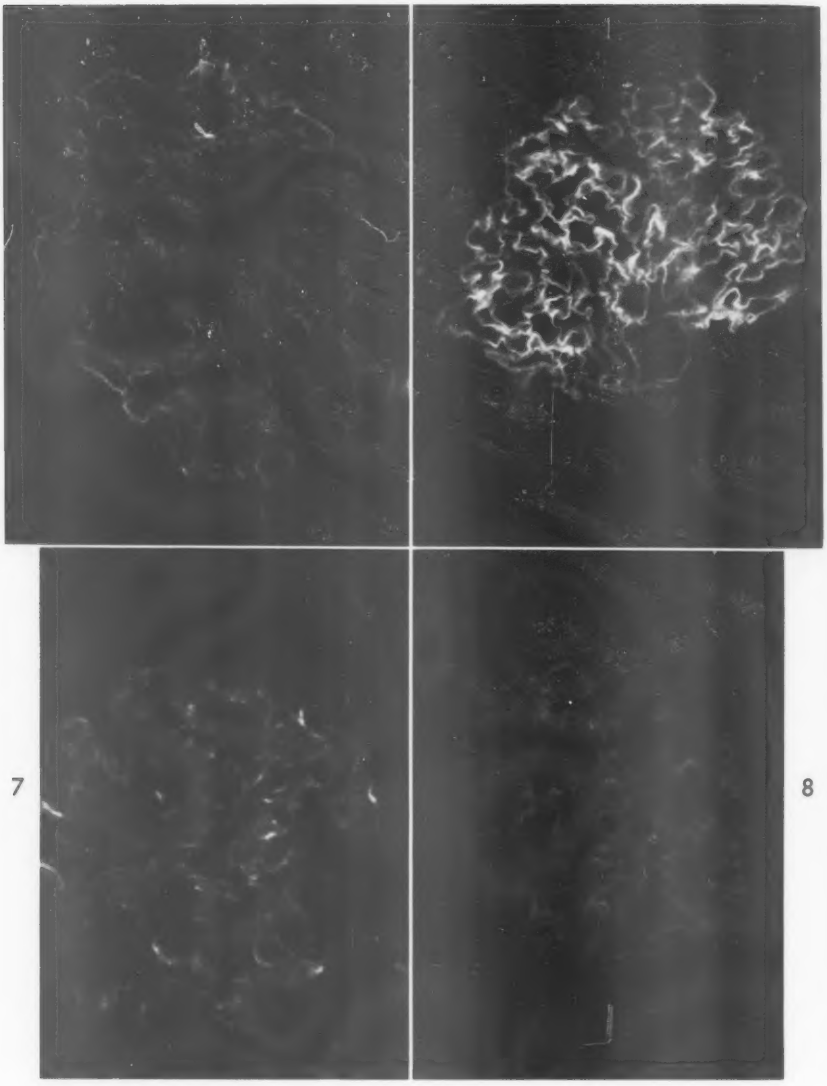


FIG. 5. RAKS injection (1 ml.), 13 days earlier. Histoserologic evidence of complement fixation with anti-complement is present. × 450.

FIG. 6. DAKS injection (1 ml.), 24 hours earlier. Stained with rabbit anti-duck globulin. DAKS appears in the basement membrane of the glomerulus. × 450.

FIG. 7. DAKS injection (1 ml.), 14 days earlier. Specific fluorescence with anti-complement appears at the site of deposition of DAKS, showing the complement fixation after a lag period. × 450.

FIG. 8. Injection of RAKS (0.5 ml.) and DAKS (1 ml.). There is no effect upon the positive complement fixation reaction. Section stained with anti-complement. × 450.

THE RENAL GLOMERULUS IN CIRRHOSIS OF THE LIVER

WALLACE A. JONES, M.D.*; D. R. GOVINDA RAO, M.B., B.S., D.P.H.,† AND HERBERT BRAUNSTEIN, M.D.‡

From the Department of Pathology, University of Cincinnati College of Medicine and the Cincinnati General Hospital, Cincinnati, Ohio

Attempts have been made in the past to discover a renal lesion associated with hepatic cirrhosis. Stimulus for this search has been provided by the observation of altered renal function [1-6] in certain forms of hepatic disease, including cirrhosis, and by provocative experimental observations relating renal and hepatic disorders. These include the simultaneous damage to liver and kidney in dogs fed alcohol while maintained on a high protein diet,[7] and the production of altered glomerular structure in animals fed a choline-deficient diet [8] or treated with estrogens.[9]

Although the existence of a specific glomerular alteration in patients with cirrhosis has been disputed, recent workers [10,11] have felt strongly that a distinctive lesion does indeed exist. One observer [11] has proposed the term "cirrhotic glomerulosclerosis," and another,[10] "membranous and proliferative glomerulonephritis of hepatic cirrhosis."

Our original purpose was to attempt a correlation between the alleged glomerular lesion of cirrhosis and the type of cirrhosis, i.e., nutritional, postnecrotic or posthepatitic.[12] To do this we examined kidney sections from 150 necropsied patients with cirrhosis, and as controls, we evaluated kidney sections from 50 comparable individuals who had no liver disease. In attempting to evaluate these tissue sections, several major technical problems became apparent. These were: great variation in apparent thickness of the sections, variation in hours post mortem and adequacy of fixation. Even under ideal conditions the routine sections from the necropsy files were difficult to evaluate quantitatively. These difficulties were emphasized when it became apparent that we were unable to separate the sections of the patients having cirrhosis from those in the control group when the slide labels were masked.

Supported in part by the John R. Stark Memorial Fund.

Accepted for publication, May 19, 1961.

* Present address: Department of Pathology, Massachusetts General Hospital, Boston 14, Mass.

† This investigation was carried out in partial fulfillment of requirements for a Master of Science degree in pathology. Present address: % Director of Public Health, Seshadri Road, Bangalore, Mysore State, India.

‡ Senior Research Fellow, United States Public Health Service.

It appeared that if a renal lesion related to cirrhosis did exist, its subtle alterations would require rigid control of technique for evaluation. Accordingly, a carefully regulated study of kidney sections taken from necropsied patients with cirrhosis and from a control group was carried out to determine whether a unique glomerular alteration was associated with hepatic cirrhosis, or whether a nonspecific alteration occurred with greater frequency in patients having cirrhosis.

Material and Methods

Necropsy sections of kidney were examined from 100 patients with unequivocal cirrhosis of the nutritional, posthepatitic or postnecrotic types,[12] and from 100 patients free of liver disease who served as controls.

In the selection of both groups, cases were excluded on the basis of any of the following features: diabetes mellitus, intrinsic inflammatory renal disorder or severe renal arterial or arteriolar disease. Cases with renal arterial lesions of minor to moderate degree were not excluded because of the almost universal occurrence of such alterations in the necropsy population at large. Twenty per cent of the patients in the group with cirrhosis and 40 per cent of those in the control group had been hypertensive with diastolic pressures above 90 mm. of Hg.

The tissue was fixed in Zenker's acetic acid or, in a few instances, in 10 per cent buffered formalin, was processed in conventional fashion and embedded in paraffin. The paraffin blocks were sectioned in an International Minot microtome at a setting of 2 μ and, in order to minimize variation in thickness, all sections were cut by the same technician, using the same microtome. One section from each case was stained with hematoxylin and eosin and another with the periodic acid-Schiff (PAS) technique.

The glomeruli in each instance were evaluated with respect to two features: alteration in basement membranes and degree of cellularity. For the study of cellularity, 30 cases of cirrhosis and an equal number from the control group were used. Nuclear counts were performed on a minimum of 10 glomeruli in each section, and the average count was taken as the index of glomerular cellularity for that section.

Significant alteration in the basement membrane appeared in the form of thickening, fraying or splitting, as observed in the sections stained with the PAS method; those that did not show any of these features were considered negative. Obsolescent glomeruli or those in small cicatrized areas were not evaluated since these changes, though of minor degree, occurred in virtually all of the cases in both groups and were clearly related to vascular sclerosis.

The sections were evaluated independently by 3 different observers, with the slide labels masked. Final revision in the few cases of disagreement was made by simultaneous study of the section by all 3 observers. Necropsy protocols of the cases were reviewed and the age, race, sex, blood pressure, heart weight and kidney weights were tabulated to determine the relation of each of these factors to the renal lesion.

Results

No difference was found in the glomerular cellularity of the two groups. The average number of cells per glomerulus was 131 in the patients with cirrhosis and 132 in the control group, with a range of 106 to 185 cells per glomerulus in the cases with cirrhosis and 101 to 176 cells in the control group.

Alteration of the basement membrane, consisting of thickening, fray-

ing or splitting, was found in over 75 per cent of the glomeruli in 28 of the 100 cases (28 per cent) with cirrhosis and in 10 of the 100 (10 per cent) controls (Table I). The basement membrane thickening was most marked in the central portion of the glomerular tuft and progressed in centrifugal fashion to affect the periphery. The alteration was focal within the glomerulus in most instances but occasionally affected the whole tuft (Figs. 1 to 4).

The degree of involvement of the glomeruli was relatively mild compared to other common varieties of glomerulosclerosis, and the slight variation in severity of the lesion made quantitative grading undesirable. The use of thin sections and the range of hours post mortem obviated critical evaluation of tubules.

TABLE I

INFLUENCE OF RACE AND SEX ON "GLOMERULOSCLEROSIS" IN CIRRHOSIS

Cirrhosis					Control (no cirrhosis)				
Sex	White	Negro	Total	%	Sex	White	Negro	Total	%
M	14/65	2/9	16/74	22	M	5/43	2/26	7/69	10
F	10/20	2/6	12/26	46	F	2/21	1/10	3/31	10
Total	24/85	4/15	28/100	28	Total	7/64	3/36	10/100	10

Male:female necropsy ratio = 1.7:1. White:Negro necropsy ratio = 2:1.
Numerator = number with lesion; denominator = total number studied.

TABLE II

RELATION OF AGE TO "GLOMERULOSCLEROSIS"

Age	20–29	30–39	40–49	50–59	60–69	70–79	80–89	Totals
Patients with cirrhosis	0/0	0/5	5/24	10/29	7/31	5/8	1/3	28/100
Controls	0/6	1/6	3/21	3/21	2/26	1/13	0/7	10/100

Numerator = number with lesion; denominator = total number studied.

TABLE III

INCIDENCE OF "GLOMERULOSCLEROSIS" RELATED TO TYPE OF CIRRHOSIS

Type of Cirrhosis	No. in group	No. with lesion	Incidence (%)
Nutritional	44	14	32
Posthepatitic	34	8	24
Postnecrotic	22	6	27

Statistical evaluation of the incidence, applying the Yates correction, revealed that the higher incidence in the cases with cirrhosis was significant, with a P value of less than 0.01, and a statistically significant ($P<0.01$) higher incidence of glomerular alteration was encountered among the women with cirrhosis (Table I).

There appeared to be an increasing incidence of glomerular alteration with advancing age among the patients with cirrhosis (Table II); however, no definite inference could be drawn since the duration of cirrhosis could not be evaluated. No significant difference was found in the incidence of the renal lesion among the 3 varieties of cirrhosis (Table III). Cardiomegaly of the hypertensive type, as judged by increase in weight of the heart without valvular disease or cor pulmonale, had no relation to the presence or absence of the renal lesion in either of the two groups, and the existence of mild to moderate hypertension was not related to the frequency of the lesion. The relation to severe hypertension was not investigated.

Review of the kidney weights revealed neither enlargement nor shrinkage in kidneys affected with glomerulosclerosis, as compared with those having no lesions.

Discussion

An alteration of the renal glomerulus was found in 28 per cent of patients dying with cirrhosis. This consisted of thickening, fraying or splitting of the basement membrane, unassociated with increased cellularity of the glomerulus. These structural features were not unique or peculiar to patients having cirrhosis, as evidenced by the occurrence of identical alterations in 10 per cent of a comparable control group of patients who had no liver disease. However, the lesion did occur with statistically significant ($P<0.01$) greater frequency among the patients with cirrhosis. The alterations, though readily detectable, were never severe, and despite variation in degree, the range of variation was minimal and did not allow a differential quantitation.

A somewhat similar structural feature affecting the glomerular basement membrane, as seen with the light microscope, appears in a number of disorders. These include diabetic glomerulosclerosis, disseminated lupus erythematosus, amyloidosis, chronic membranous glomerulonephritis associated with the nephrotic syndrome (lipoid nephrosis) and hypertensive vascular disease. However, the renal alterations found in this study did not approach the severity of those usually seen in other diseases involving the basement membrane. They did, however, resemble the early glomerular alterations seen in association with arterionephrosclerosis. In this regard, lesions of the glomerulus in arterionephrosclerosis, described by Kimmelsteil[13] in 1935, are essentially identical with those found by us in the "positive" cases in the present investigation.

Several studies[14–16] have dealt with renal lesions associated with liver disease in general, but apparently the first concerned with those

in cirrhosis was that of Baxter and Ashworth[17] in 1946. These authors investigated 25 necropsied patients with cirrhosis, and although tubular alterations were observed, no glomerular lesions that could not be accounted for on the basis of renal vascular disease were detected. No controls were reported in this study. In 1951, Patek, Segal and Bevans[18] stated that in 11.7 per cent of 60 patients with cirrhosis, glomerulonephritis of the membranous type was found at necropsy. These lesions were not illustrated, and no control group was recorded. Fisher and Hellstrom,[10] in 1959, stated "a distinctive, but not specific, glomerular lesion characterized by fibrillary thickening of the endothelial stalks and occasionally basement membranes of capillary loops was encountered in 25 per cent of 100 consecutive necropsied cases of hepatic cirrhosis and 1 of 2 examples of hemochromatosis." Although it was indicated that control cases were examined, only the glomerular cell counts of the two groups were compared. Basement membrane alteration in the control cases was not mentioned. Bloodworth and Sommers[11] examined 100 cases of cirrhosis and found "glomerulosclerosis" in 78 per cent. The thickness of the tissue sections was not indicated, and no control group was offered for comparison.

Glomerular alterations have been observed by others in patients with cirrhosis, but lack of uniformity in tissue preparation and lack of comparable control groups makes interpretation of their results difficult. Early in our study it became apparent that uniformly thin tissue sections would be required to evaluate basement membranes critically. It was noted, for example, that with the same microtome setting, the thickness of sections varied widely with different technicians. Our investigation was, therefore, carried out upon sections cut by a single skilled technician using the same microtome. Under such stringently controlled conditions, identical glomerular basement membrane alterations were detected in both cirrhotic and noncirrhotic groups.

Our findings stand in contrast to those of other investigators mainly in the demonstration of the lesion in patients without cirrhosis, and the observation of a much lower incidence in patients with cirrhosis than reported in one of the two most recent investigations.[11] The same basic lesion has probably been observed by all investigators. Many similarities can be seen in the "intercapillary glomerulonephritis" of Patek and coworkers,[18] the "cirrhotic glomerulosclerosis" of Bloodworth and Sommers,[11] and the "membranous and proliferative glomerulonephritis" of Fisher and Hellstrom.[10] The varied incidence reported by different authors probably resulted from technical problems and the misleading appearance of the "basement membrane" in hematoxylin and eosin (H and E) stained sections. In many instances it was found that basement mem-

branes interpreted as thickened in the H and E sections were actually delicate and quite normal when examined in sections stained by the PAS method (Figs. 5 and 6). This problem was emphasized in another study by one of us (W.A.J.),[19] in which kidney sections were examined at necropsy in 9 young women who in life had had hyperglobulinemia (globulin, 3.7 to 11.2 gm. per cent) and either postnecrotic cirrhosis or subacute hepatic necrosis. In 4 of the patients H and E stained sections were deceptive in presenting broadened eosinophilic bands that obscured the normal underlying basement membrane; the latter proved to be normal in the PAS sections in all 9 cases. The apparent thickening seen in H and E stained preparations may reflect the deposition of protein in the cytoplasm of podocytes. Recent electron microscopic studies[20] support this concept.

The etiology of the basement membrane alterations in our "positive" cases remains speculative. However, certain clues regarding influencing factors are provided in the correlative data. The frequency of the lesion tended to increase with advancing age in the patients with cirrhosis and occurred more frequently in the female patients with cirrhosis. The only consistent factor in the "positive" cases in both groups was arterionephrosclerosis, and this lesion would seem to be necessary for the development of the alteration. In experimental hypertension, however, Dammin, Goldman, Schroeder and Pace[21] found glomerular basement membrane alterations preceding the development of arterial and arteriolar sclerosis.

It is possible that cirrhosis, as a chronic disease, may influence the production of the lesion in a nonspecific way. On the other hand, several other possibilities are suggested by certain experimental and pathologic observations. The long-continued administration of stilbestrol to guinea pigs[9] resulted in glomerular changes consisting of cellular proliferation and the deposit of a PAS-positive laminated extracellular substance evenly distributed in the "intercapillary spaces" of the tuft. This produces narrowing of the capillary lumen that very likely results in ischemia. Similar changes have been reported[9] in men with prostatic cancer, receiving stilbestrol therapy, and in the glomeruli of pregnant women, some of whom died of eclampsia,[22] others[23] of a variety of conditions. Perhaps the impaired inactivation of estrogen in patients with cirrhosis is responsible for the production or accelerated rate of development of the basement membrane aberration. The greater availability of estrogen in the female may explain the increased frequency of the lesion in women with cirrhosis.

Nutritional factors have been suggested by the simultaneous production of hepatic and renal lesions in rats fed choline-deficient diets.[8]

However, these structural alterations consisted of hemorrhagic necrosis in both organs and were quite unlike those found in the present study. In cirrhosis induced by choline deficiency, fat emboli have been demonstrated [24] in a number of organs, including the kidney. Allegedly, renal fat embolism stems from fatty hepatic "cysts." The resulting obstruction of glomerular circulation is believed to lead to focal necrosis and tuftal hyalinization. In many of the patients with cirrhosis in our study the livers were nonfatty, and in those with fatty nutritional cirrhosis, glomerular necrosis was not found.

Electron microscopic [8] examination of renal changes in choline-deficient rats has revealed parenchymatous degeneration, swelling of glomerular capillary walls and necrosis of proximal convoluted tubules. It was suggested that glomerular capillary alteration might have been associated with abnormalities in permeability to protein. Recent work [25] has rather convincingly demonstrated that the renal injury which occurs in acute choline deficiency is the result of ischemia attributable to increased responsiveness of arterioles and increased vascular fragility. Investigation [7] in dogs fed alcohol and a high protein diet revealed damage of both liver and kidney, suggesting that changes in renal circulation mediated, either directly or indirectly, by alcohol were important in the induction of the renal lesion.

Many other mechanisms have been proposed to explain renal lesions associated with liver disease. In the early investigations by Boyce and McFetridge [15] in cases of "liver death," renal tubular degeneration and necrosis but no glomerular alterations were found. It was suggested that the tubular damage was brought about by the renal excretion of an unidentified "toxic substance" elaborated by damaged liver cells. In a study of "toxic oliguria" and lower nephron nephrosis, French [26] found glomerular and tubular alterations in addition to liver damage and suggested that the failure of the liver to deaminate amino acids and other products of protein catabolism resulted in the excretion of substances toxic to the kidney. Crowson and More [14] suggested that in cases of so-called "glomerulotubular nephrosis" associated with hepatic disease "vasopressor materials accumulate in the systemic circulation during periods of hepatic insufficiency and produce renal ischemia."

Although the glomerular alterations in our cases were probably related to arteriosclerosis and ischemia, the cause of the increased incidence in the patients with cirrhosis remains an enigma. The renal lesions would not seem to explain the alterations of renal function reported in patients with cirrhosis. In addition to the common features of albuminuria [5] and cylindruria, some [1] have found increases in glomerular filtration rate, renal plasma flow and filtration fractions. However, others [4]

have observed normal creatinine clearance with decreased diuretic response to water loading and a diminished maximal rate of sodium excretion in "compensated" patients with cirrhosis. Most of the patients have had a normal glomerular filtration rate and renal plasma flow, accompanying impaired renal excretion of sodium and water. It has been postulated that decreased effective extracellular fluid volume stimulates increased tubular reabsorption of sodium and the major factor limiting water excretion is reduction of sodium excretion.

The mediating factors responsible for the altered renal function may be related to abnormal adrenal metabolism. Bongiovanni and Eisenmenger[27] found a depressed daily excretion of 17-ketosteroids and an elevation of corticoids in patients with "chronic hepatic disease"; more recent studies[28,29] have demonstrated increased urinary levels of aldosterone in patients having cirrhosis with ascites.

A recent report by Papper, Belsky and Bleifer[30] concerned 22 patients with alcoholism and cirrhosis who died in renal failure. It was proposed here that a reduction of glomerular filtration rate and a diminution of renal blood flow were responsible for the renal failure rather than renal parenchymal disease.

Summary

In order to determine whether a specific renal lesion was associated with cirrhosis, a study of kidney sections was carried out in 100 patients with nutritional, posthepatitic or postnecrotic cirrhosis and in 100 patients without liver disease who served as controls. Emphasis was placed on strict control of section preparation, and sections 2 μ thick, stained by the PAS technique were utilized.

A basement membrane alteration consisting of splitting, fraying or thickening was found in 28 per cent of the cases with cirrhosis and in 10 per cent of the controls. There was no difference in the glomerular cellularity in the two groups. The basement membrane lesion occurred with approximately the same frequency in each of the 3 types of cirrhosis.

Three other factors appeared to influence the occurrence of the lesion. Arterionephrosclerosis was a constantly accompanying feature. The incidence of this process increased with advancing age in patients with cirrhosis. Among patients with cirrhosis, women had a higher incidence of glomerular change. The mechanisms by which advancing age, sex, and cirrhosis operate to increase the incidence of the basement membrane alteration remain obscure.

The rate of occurrence of the lesion and its nonspecific nature, as demonstrated in this study, differ from the results in previous investiga-

tions and probably reflect the utilization of both rigid controls and a double blind technique.

References

1. Duque, I.; Jimenez, I., and Latorre, G. Renal function in liver disease and malnutrition. *Am. J. M. Sc.*, 1959, **237**, 722–726.
2. Popper, H., and Schaffner, F. Liver: Structure and Function. Blakiston Div., McGraw-Hill Book Co., Inc., New York, Toronto, London, 1957, p. 294.
3. Papper, S. The role of the kidney in Laennec's cirrhosis of the liver. *Medicine*, 1958, **37**, 299–316.
4. Papper, S., and Rosenbaum, J. D. Abnormalities in the excretion of water and sodium in "compensated" cirrhosis of the liver. *J. Lab. & Clin. Med.*, 1952, **40**, 523–530.
5. Morrison, L. M. Improvement in kidney function following treatment for cirrhosis of the liver. *Rev. Gastroenterol.*, 1947, **14**, 533–538.
6. Hecker, R., and Sherlock, S. Electrolyte and circulatory changes in terminal liver failure. *Lancet*, 1956, **2**, 1121–1125.
7. Chaikoff, I. L.; Entenman, C.; Gillman, T., and Connor, C. L. Pathologic reactions in the livers and kidneys of dogs fed alcohol while maintained on a high protein diet. *Arch. Path.*, 1948, **45**, 435–446.
8. Ashworth, C. T., and Grollman, A. Electron microscopy in experimental hypertension. Glomerular changes in choline-deficiency-induced hypertension in the rat. *Arch. Path.*, 1959, **68**, 148–153.
9. Trevan, D. Glomerular changes induced by stilbestrol; preliminary communication. *Lancet*, 1956, **2**, 22–23.
10. Fisher, E. R., and Hellstrom, H. R. The membranous and proliferative glomerulonephritis of hepatic cirrhosis. *Am. J. Clin. Path.*, 1959, **32**, 48–55.
11. Bloodworth, J. M. B., Jr., and Sommers, S. C. "Cirrhotic glomerulosclerosis," a renal lesion associated with hepatic cirrhosis. *Lab. Invest.*, 1959, **8**, 962–978.
12. Gall, E. A. Posthepatitic, postnecrotic and nutritional cirrhosis; a pathologic analysis. *Am. J. Path.*, 1960, **36**, 241–271.
13. Kimmelstiel, P. Glomerular changes in arteriosclerotic contraction of the kidney. *Am. J. Path.*, 1935, **11**, 483–496.
14. Crowson, C. N., and More, R. H. Glomerulotubular nephrosis correlated with hepatic lesions. II. Incidence and morphology of associated kidney and liver lesions in human autopsy material. *Arch. Path.*, 1955, **60**, 73–84.
15. Boyce, F. F., and McFetridge, E. M. So-called "liver death"; a clinical and experimental study. *Arch. Surg.*, 1935, **31**, 105–136.
16. Ayer, D. Renal lesions associated with deep jaundice; with comments on their relations to those in the so-called hepatorenal syndrome and in transfusion reactions. *Arch. Path.*, 1940, **30**, 26–41.
17. Baxter, J. H., and Ashworth, C. T. Renal lesions in portal cirrhosis. *Arch. Path.*, 1946, **41**, 476–488.
18. Patek, A. J., Jr.; Seegal, D., and Bevans, M. The coexistence of cirrhosis of the liver and glomerulonephritis; report of 14 cases. *Am. J. M. Sc.*, 1951, **221**, 77–85.
19. Jones, W. A., and Castleman, B. An anatomic study of the liver in young women with liver disease and hyperglobulinemia. (In preparation)
20. Post, R. S. The effects on glomerular structure of proteinuria induced in nor-

mal rats determined by electron microscopy. Proceedings of the Eleventh Annual Conference on the Nephrotic Syndrome, 1959. METCOFF, J. (ed.) National Kidney Disease Foundation, New York, 1960, pp. 222–228.

21. DAMMIN, G. J.; GOLDMAN, M. L.; SCHROEDER, H. A., and PACE, M. G. Arterial hypertension in dogs. II. The effects of neurogenic hypertension with a study of periodic renal biopsies over a seven-year period. *Lab. Invest.*, 1956, **5**, 72–96.

22. BAIRD, D., and DUNN, J. S. Renal lesions in eclampsia and nephritis of pregnancy. *J. Path. & Bact.*, 1933, **37**, 291–309.

23. GOVAN, A. D. T. Renal changes in eclampsia. *J. Path. & Bact.*, 1954, **67**, 311–322.

24. HARTROFT, W. S., and RIDOUT, J. H. Pathogenesis of the cirrhosis produced by choline deficiency; escape of lipid from fatty hepatic cysts into the biliary and vascular systems. *Am. J. Path.*, 1951, **27**, 951–989.

25. FULTON, L. A., and LEE, R. E. Acute choline deficiency in albino rats: vascular contractility, vascular fragility and blood pressure. *Proc. Soc. Exper. Biol. & Med.*, 1958, **97**, 288–291.

26. FRENCH, A. J. Glomerulonephrosis: a morphologic manifestation of renal cortical ischemia in toxic oliguria and lower nephron nephrosis. *Arch. Path.*, 1950, **49**, 43–54.

27. BONGIOVANNI, A. M., and EISENMENGER, W. J. Adrenal cortical metabolism in chronic liver disease. *J. Clin. Endocrinol.*, 1951, **11**, 152–172.

28. GORNALL, A. G.; GWILLIAM, C., and HALL, A. E. D. Aldosterone excretion in various clinical states. (Abstract) *J. Clin. Endocrinol.*, 1956, **16**, 950.

29. DYRENFURTH, I.; STACEY, C. H.; BECK, J. C., and VENNING, E. H. Aldosterone excretion in patients with cirrhosis of the liver. *Metabolism*, 1957, **6**, 544–555.

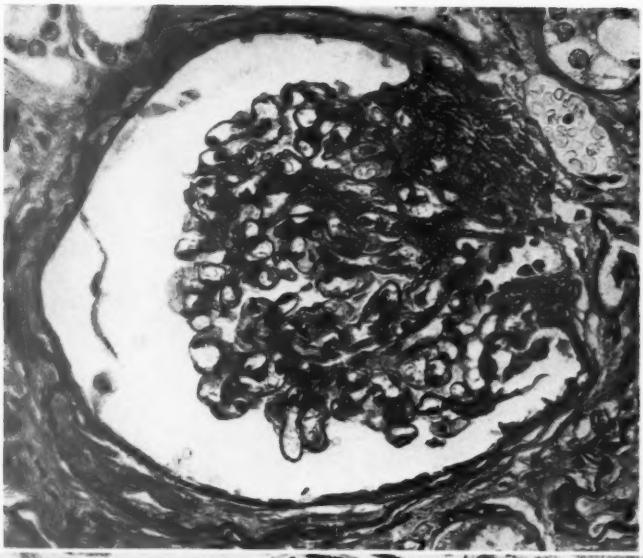
30. PAPPER, S.; BELSKY, J. L., and BLEIFER, K. H. Renal failure in Laennec's cirrhosis of the liver. I. Description of clinical and laboratory features. *Ann. Int. Med.*, 1959, **51**, 754–773.

Mrs. Helena Johnson, research assistant, prepared the histologic sections. Her invaluable aid is hereby acknowledged with appreciation.

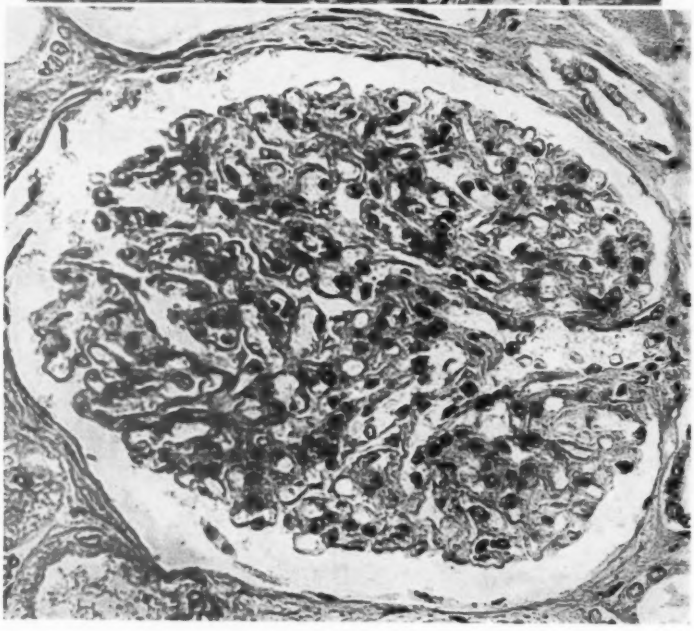
LEGENDS FOR FIGURES

FIG. 1. A representative glomerulus in a case of cirrhosis. There is apparent thickening and fraying of the basement membrane; an adhesion is evident near the hilus. Periodic acid-Schiff (PAS) stain; section 2 μ thick. × 325.

FIG. 2. A glomerulus from the same case shown in Figure 1. The basement membrane alteration appears less marked. There is suggestive but not conclusive evidence of thickening. Hematoxylin and eosin stain; section 2 μ thick. × 325.



1



2

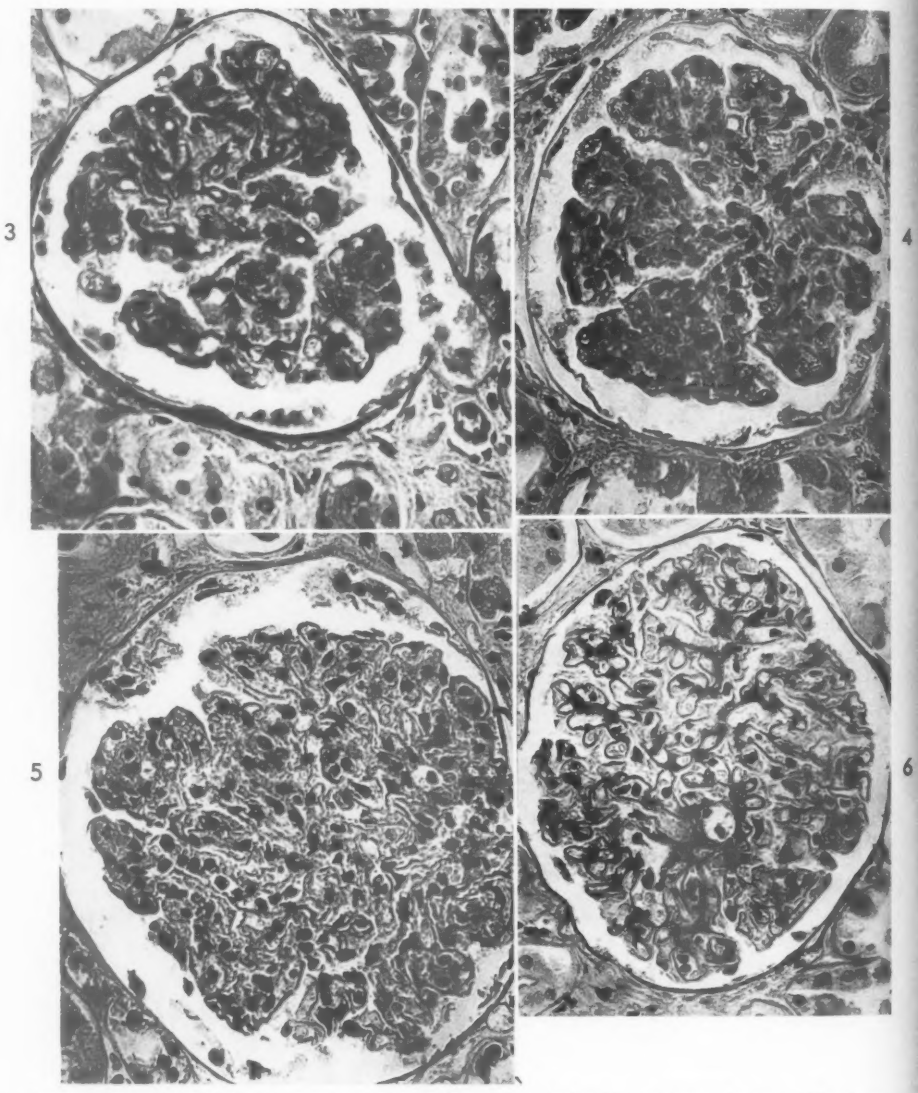


FIG. 3. A representative glomerulus in the kidney of an individual without cirrhosis. The basement membrane is irregularly frayed and thickened. PAS stain, section 2 μ thick. $\times$ 325.

FIG. 4. A comparable glomerulus from the section portrayed in Figure 3. Alterations are similar. Hematoxylin and eosin stain; section 2 μ thick. $\times$ 325.

FIG. 5. A "routine" kidney section in an instance of cirrhosis. There is suggestive evidence of glomerulosclerosis. Hematoxylin and eosin stain; section "5 μ" thick. $\times$ 325.

FIG. 6. A "thin" section from the same kidney shown in Figure 5. The glomerular basement membrane here appears normal. PAS stain; section 2 μ thick. $\times$ 325.

FIXED AND REPRODUCIBLE ORTHOSTATIC PROTEINURIA

II. Electron Microscopy of Renal Biopsy Specimens from Five Cases

Captain Roscoe R. Robinson, MC, USAF *; Charles T. Ashworth, M.D.;
Captain Sorrell N. Glover, MC, USAF †; Captain Paul J. Phillippi, MC, USAF;
Captain Frank R. Lecocq, MC, USAF,
and Lieutenant Colonel Paul R. Langelier, MC, USAF

From the USAF Hospital, Lackland, USAF Aerospace Medical Center (ATC), Lackland Air Force Base, Texas, and the Department of Pathology, University of Texas, Southwestern Medical School, Dallas, Texas

In a previous light microscopic study, characteristic glomerular alterations were observed in 53 per cent of renal biopsy specimens from 56 asymptomatic patients with fixed and reproducible orthostatic proteinuria.[1] These changes consisted primarily of enlargement of the glomerular tuft with foci of hypercellularity, slight capillary wall thickening, and decreased prominence of the capillary lumens, focal capillary dilatation, and clusters of eosinophilic and PAS-positive droplets in close proximity to the epithelial periphery of the glomerular tuft. Since these alterations were of a minimal nature, it was considered desirable to determine whether additional fine structural changes not seen with conventional microscopy might be found. Accordingly, renal biopsy specimens from 5 patients with fixed and reproducible orthostatic proteinuria were examined by electron microscopy.

Material and Methods

Five young men (ages 17 to 18 years) with fixed and reproducible orthostatic proteinuria were selected according to criteria described elsewhere [1] and subjected to percutaneous renal biopsy. For control purposes, one renal biopsy was made in a young male volunteer patient (age 22 years) who was free of both proteinuria and evidence of renal disease. Biopsy samples were sliced into two equal halves along their longitudinal axes; one portion was placed immediately into 10 per cent neutral buffered formalin in preparation for light microscopy; the other half was cut into small blocks, 1 cu. mm. or less in size, and placed in chrome-osmium fixative [2] for electron microscopy. Formalin-fixed tissue was embedded in paraffin, sectioned with a con-

This investigation was supported in part by Research Contract 41(657)-368 with the School of Aviation Medicine, USAF Aerospace Medical Center (ATC), Brooks Air Force Base, Texas, and partly by United States Public Health Service grant No. H-5120 to one of us (C.T.A.).

The opinions expressed in this paper are those of the authors, and are not to be construed as an official statement of Air Force policy.

Accepted for publication, May 2, 1961.

* Present address: Department of Medicine, Duke University Medical Center, Durham, N.C.

† Present address: Department of Pathology, University of California Medical Center, Los Angeles, Calif.

ventional microtome, and stained with hematoxylin and eosin and the periodic acid-Schiff (PAS) reagent. Tissue for electron microscopy was dehydrated in graded alcohols, embedded in methacrylate, sectioned with a diamond knife, and examined with an RCA EMU-3 electron microscope. "Thick" methacrylate sections (0.5 to 1 μ) were also prepared with May-Grünwald stain for parallel light microscopic study of tissue subjected to electron microscopy. From 4 to 15 glomeruli were examined per biopsy with conventional light microscopy. In each case, an average of 3 glomeruli was evaluated with the electron microscope.

Observations

Light microscopy of sections from 5 patients with fixed and reproducible orthostatic proteinuria revealed that about 25 per cent of the glomeruli exhibited histologic changes similar to those described previously.[1] For purposes of comparison, a representative glomerulus from the normal subject (Fig. 1) is shown beside a glomerulus from a patient with proteinuria (Fig. 2) which exhibits certain of the histologic features[1] found in that condition.

Multiple focal alterations of the glomeruli were also found with electron microscopy. In each of the cases with proteinuria, about one third of the glomeruli contained foci with collapsed capillary lumens, relatively increased cellularity, and irregularly folded condensations of basement membrane substance. Widely patent capillary lumens frequently were noted adjacent to areas of capillary obliteration. Alterations of this type appeared to correspond to the foci of hypercellularity seen in the glomerular tufts with the light microscope.[1] The structural pattern of these changes differed from that produced by tangential sections of normal capillary walls which also were found in the normal subject (Fig. 3). Focal areas of capillary wall alteration were distinguished clearly in cases with proteinuria by their more extensive nature, more frequent occurrence, and by the involvement of several adjoining capillary loops (Figs. 4 and 5).

One of the most striking glomerular features consisted of "watery" cytoplasmic swelling in the epithelium of capillary walls. Whereas the normal epithelium had a dense and compact cytoplasm containing relatively few vacuoles (Fig. 3), the epithelium in most of the glomeruli in patients with proteinuria contained an increased amount of cytoplasm, within which vesicles were more numerous and ribonucleoprotein granules and other cytoplasmic constituents were more widely dispersed (Fig. 6). In some instances, sheet-like groupings of large epithelial cells were noted at the periphery of the glomerular tufts. Many of these cells demonstrated microvilli and infoldings of the cell membrane on their free surfaces; the latter in many respects resembled renal tubular epithelium (Fig. 7). Evidence of pinocytosis, consisting of small invaginations of the cell membrane, was observed with greater frequency

and was more marked in the cases of proteinuria than in the normal subject. The swollen epithelium may have been an important cause of the capillary wall thickening seen in hematoxylin and eosin stained preparations in patients with proteinuria.[1]

Focal foot process fusion was frequently observed (Fig. 8). The adjacent capillary lumens were usually patent. In many instances minute infoldings or notches were seen along the surfaces of the fused foot processes, where they directly adjoined the outer aspect of the basement membrane proper (Fig. 9). These notches appeared to represent the remnants of interpedicel spaces which had been almost completely obliterated. Usually, the epithelial cytoplasm was light and "watery" in appearance at the sites of foot process fusion. However, small areas with marked electron density were frequent, and they were disposed in such a way as to suggest the remains of original dense pedicel substance. Although loss of foot process organization was observed in all glomeruli, it was distinctly focal in nature and only a small portion of the total capillary surface was affected.

In 4 of the 5 proteinuria specimens, dense aggregates were found within the cytoplasm of the swollen glomerular epithelium. These appeared to be represented by various stages of development. The earliest or least advanced stage consisted of a poorly outlined collection of granular material coalescing within the cytoplasm to form a vague density (Fig. 10). Further stages of the same process were recognized, in which the granules had become more compactly arranged. At this point, within the epithelial cytoplasm, well-delineated round or oval densities were formed which ultimately appeared almost homogeneous in nature (Fig. 11). In the final formative stage, the masses measured 1 to 2 μ in diameter and closely resembled the hyaline droplets described previously within the capsular space and proximal convoluted tubular epithelium.[1] It should be pointed out that these areas of electron density differed from the discrete "spherical bodies" of glomerular epithelium described by Bergstrand and Bucht in normal human renal biopsy material.[3] The "spherical bodies" are more discrete and homogeneous and are even more dense than the aggregates found in patients with proteinuria. Bodies of this type were found also in tissue from both normal patients and those with proteinuria.

Electron microscopy failed to demonstrate overt alteration of the fine structure of the basement membrane substance. It was occasionally possible to visualize a fine fibrillar network comprising the membrane (Fig. 12). In addition, the inner surface of portions of it was quite irregular, "split," or frayed in 4 cases of proteinuria (Fig. 13). It contained many small strands and particles of moderately dense material

which were only partially continuous with the main basement membrane structure. The nature of this material was indeterminate; in density and continuity of structure, it appeared to represent imperfect deposition of basement membrane substance in the zone between the endothelial lining and the main structure of the basement membrane.

The study of tubular architecture in these cases revealed relatively few alterations. A small number of round and oval-shaped dense bodies were observed in the epithelial cytoplasm of the proximal convoluted tubules. These bodies corresponded to the PAS-positive droplets seen in the proximal convoluted tubules and appeared to be identical with the hyaline droplet material. Other round and discrete bodies, 1 to 3 μ in diameter, were also observed in the proximal tubular epithelial cytoplasm and appeared to consist of collections of dense cytoplasmic material and numerous small mitochondria.

Discussion

In renal biopsy specimens from 5 patients with fixed and reproducible orthostatic proteinuria, electron microscopy confirmed the presence of previously described glomerular alterations [1] and extended the observations on their nature. Small foci of glomerular alteration were found in all 5 cases with both light and electron microscopy.

Electron microscopy demonstrated that foci of hypercellularity observed with the light microscope [1] consisted of collapsed and completely or partially obliterated capillary loops with increased and irregular basement membrane deposits and, in some instances, swollen endothelial cells within the partially collapsed capillary lumens. In many respects, these alterations were similar to those seen in acute glomerulonephritis [4] and may have been responsible for permanent obliteration of the capillary lumens at these points. The existence of more widely dilated capillaries in adjacent portions of the glomerular tuft perhaps may be attributed to local increases in blood flow provoked by focal narrowing and obliteration of capillaries. The electron microscopic features suggested that an active lesion focally affecting the glomerular capillary loops of these patients may have been present in the past. The exact nature of this disorder was not revealed by the present study. It resembled that described as "persistent focal nephritis" [5] in many ways. It is natural to speculate that the initial focal lesion was inflammatory in type and that it was associated with capillary endothelial swelling and obliteration of the lumen. Viewed in this manner, the alterations noted in the present series of specimens may have represented the end or healing stages of an early bout of either mild and focal proliferative or membranous glomerulonephritis. Other varieties of past glomerular injury,

either chemical or metabolic in nature, cannot be excluded, however. Similarly, the eventual clinical course of patients with glomerular alterations of this type cannot be anticipated.

The character and distribution of the glomerular alterations suggest that protein loss probably takes place from focal sites in the altered glomeruli. This may be contrasted with the diffuse loss of protein which occurs in more severe proteinuric or nephrotic states. It is probable that most other areas of the glomerular capillary walls remain relatively impermeable to protein. This may account for the mild protein loss seen in orthostatic proteinuria, and for its dependence upon the normal renal hemodynamic changes which occur on standing.[1,6,7]

In addition to spotty obliteration of the capillary loops, a second group of focal glomerular alterations was found in these cases. These consisted of foot process fusion and glomerular epithelial cytoplasmic swelling. These changes resembled those described in lipoid nephrosis[8–10] and other proteinuric states[11] except for their limited extent and focal nature.

The genesis of foot process fusion seemed to be revealed by the study of the small focal lesions. The presence of tiny remnants of interpedicel spaces suggested that fusion was produced by an "ironing out" or obliteration of the interpedicel spaces subsequent to epithelial cytoplasmic swelling. A similar genesis of foot process fusion has been suggested by the electron microscopic investigation of other forms of proteinuria in both human and experimental nephropathies.[12,13] According to this explanation, foot process fusion is interpreted as a structural alteration secondary to swelling of the epithelium itself, and therefore it is not a primary factor responsible for excessive protein loss.

The increased number of intracytoplasmic vesicles in the glomerular epithelium is of considerable interest in these cases. It suggests that these cells are engaged in the imbibition of glomerular filtrate and related macromolecules by pinocytosis.

There is strong evidence that glomerular epithelium normally takes up both fluid and macromolecules from the glomerular filtrate.[14,15] One would thus expect an increased pinocytotic uptake of macromolecules in the event of the escape of an excessive amount of protein into the glomerular filtrate. Protein taken up in this manner should be aggregated within the epithelial cytoplasm. This is the probable route and means of development of the dense cytoplasmic bodies described in the glomerular epithelium of patients with proteinuria. Ultimately, these aggregates lead to the formation of hyaline protein droplets. A similar mechanism seems to be operative in all cases of human and experimental proteinuria which have been studied.[12,13] In addition, the excessive ac-

cumulation of water and protein in epithelium by pinocytosis results in generalized cellular swelling and the development of a "watery" type of cytoplasm.

An analysis of the glomerular alterations described in fixed and reproducible orthostatic proteinuria indicates that this disorder is glomerular in origin. Unfortunately, the precise cause and mechanism of the increased protein transfer across the glomerular capillary wall has not been determined. It is logical to assume that it is related to a focal increase in the permeability of the glomerular basement membrane. However, we have been unable to demonstrate an obvious structural defect in the composition of this structure which would adequately account for such an increase in permeability to protein.

Summary and Conclusion

Electron microscopy of renal biopsy specimens from 5 patients with fixed and reproducible orthostatic proteinuria disclosed focal glomerular alterations which corresponded to those encountered with light microscopy. These consisted of small foci with collapsed glomerular capillaries, hypercellularity of glomerular loops, and increased deposition of basement membrane substance. The focal lesions were interpreted as the probable sequel of a previous episode of mild and focal acute inflammation in the glomeruli with endothelial swelling and capillary obliteration.

In addition, focal foot process fusion was noted in association with swelling of the glomerular epithelial cytoplasm, an increased number of pinocytotic vesicles, and a cytoplasmic accumulation of dense proteinoid aggregates. These epithelial changes were felt to be the result of an increased pinocytotic uptake of protein-containing fluid comprising the glomerular filtrate.

Although many of the glomeruli contained definite alterations, they were usually mild and focal in nature. The fine structure of many glomerular capillaries was entirely normal. We concluded that the excessive loss of protein in fixed and reproducible orthostatic proteinuria was the result of a focal defect in the basement membrane proper, an alteration which was not yet anatomically evident. This defect permitted an increased transfer of protein across the glomerular capillary wall following normal and unimpaired renal hemodynamic responses occurring on the assumption of an upright position.

References

1. Robinson, R. R.; Glover, S. N.; Phillippi, P. J.; Lecocq, F. R., and Langelier, P. R. Fixed and reproducible orthostatic proteinuria. I. Light microscopic studies of the kidney. *Am. J. Path.*, 1961, **39**, 291–306.
2. Dalton, A. J. A chrome-osmium fixative for electron microscopy. (Abstract) *Anat. Rec.*, 1955, **121**, 281.

3. BERGSTRAND, A., and BUCHT, H. Anatomy of the glomerulus as observed in biopsy material from young and healthy human subjects. *Ztschr. Zellforsch.*, 1958, **48**, 51–73.

4. FARQUHAR, M. G.; VERNIER, R. L., and GOOD, R. A. An electron microscopic study of the glomerulus in nephrosis, glomerulonephritis and lupus erythematosus. *J. Exper. Med.*, 1957, **106**, 649–660.

5. ROSS, J. H. Recurrent focal nephritis. *Quart. J. Med.*, 1960, **29**, 391–406.

6. KING, S. E. Patterns of protein excretion by the kidneys. *Ann. Int. Med.*, 1955, **42**, 296–323.

7. KING, S. E., and BALDWIN, D. S. Renal hemodynamics during erect lordosis in normal man and subjects with orthostatic proteinuria. *Proc. Soc. Exper. Biol. & Med.*, 1954, **36**, 634–636.

8. FARQUHAR, M. G.; VERNIER, R. L., and GOOD, R. A. Studies on familial nephrosis. II. Glomerular changes with the electron microscope. *Am. J. Path.*, 1957, **33**, 791–817.

9. MOVAT, H. Z., and MCGREGOR, D. G. The fine structure of the glomerulus in membranous glomerulonephritis (lipoid nephrosis) in adults. *Am. J. Clin. Path.*, 1959, **32**, 109–127.

10. FOLLI, G.; POLLAK, V. E.; REID, R. T. W.; PIRANI, C. L., and KARK, R. M. Electron-microscopic studies of reversible glomerular lesions in the adult nephrotic syndrome. *Ann. Int. Med.*, 1958, **49**, 775–795.

11. SPIRO, D. The structural basis of proteinuria in man. Electron microscopic studies of renal biopsy specimens from patients with lipid nephrosis, amyloidosis, and subacute and chronic glomerulonephritis. *Am. J. Path.*, 1959, **35**, 47–73.

12. ASHWORTH, C. T., and JAMES, J. A. Electron microscopy of renal biopsies in membranous glomerulonephritis in children. (To be submitted)

13. HESS, E., and ASHWORTH, C. T. Electron microscopic study of auto-immune nephrosis in rats. (To be submitted)

14. FARQUHAR, M. G., and PALADE, G. E. Segregation of ferritin in glomerular protein absorption droplets. *J. Biophys. & Biochem. Cytol.*, 1960, **7**, 297–304.

15. JAMES, J. A., and ASHWORTH, C. T. Some features of glomerular filtration and permeability revealed by electron microscopy after intraperitoneal injection of dextran in rats. *Am. J. Path.*, 1961, **38**, 515–525.

The helpful cooperation of Mr. Emil Sanders in the Department of Pathology, University of Texas, Southwestern Medical School, in the electron microscope studies, is gratefully acknowledged.

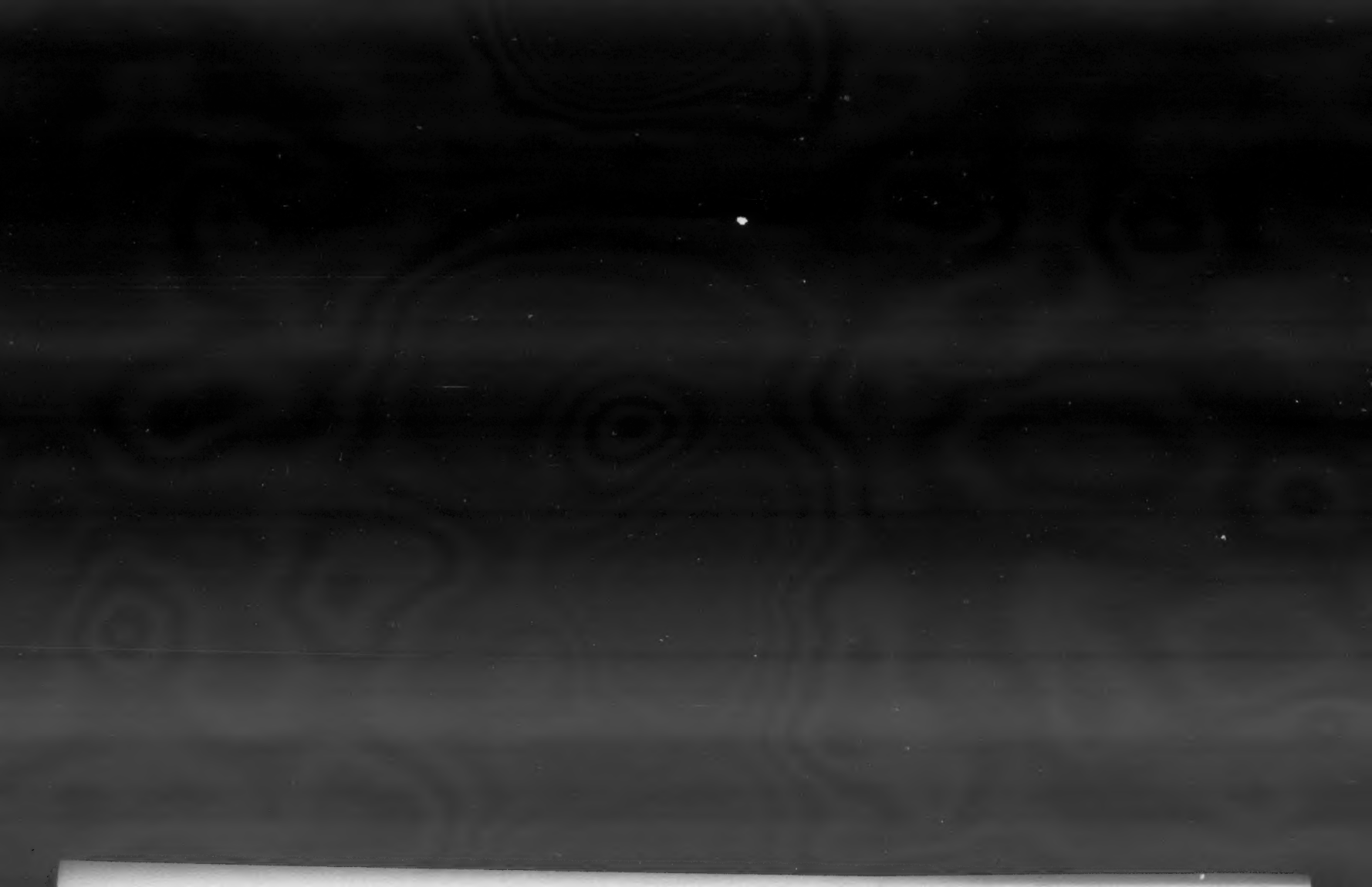
[*Illustrations follow*]

Legends for Figures

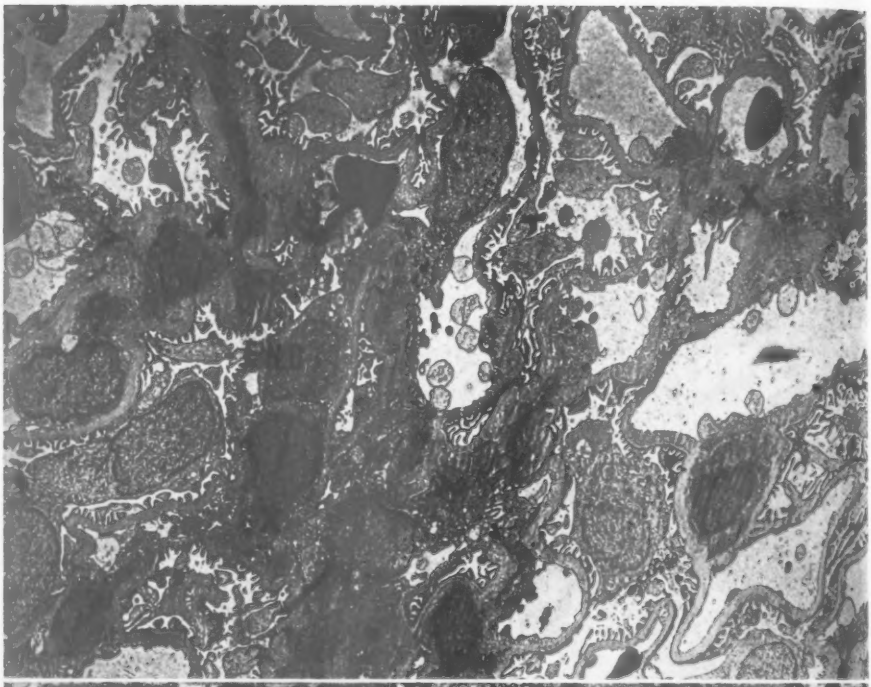
FIG. 1. Glomerulus in a normal patient. Patent and discrete capillaries with delicate basement membrane are visible. Periodic acid-Schiff (PAS) stain. × 285.

FIG. 2. Glomerulus in a patient with orthostatic proteinuria (case 4). Focal collapse and adherence of capillary loops with thickened basement membranes are seen (X). PAS stain. × 285.

FIG. 3. Portion of a glomerulus from a normal patient. The parietal layer of Bowman's membrane (P) is shown. Capillary loops (C) are patent and are not adherent. At "X" the capillaries are partly sectioned through the walls, and lumens are either small or cannot be seen. The glomerular epithelial cells (E) are relatively small and their cytoplasm is compact. Foot processes (F) are well formed. × 3,000.



4



5

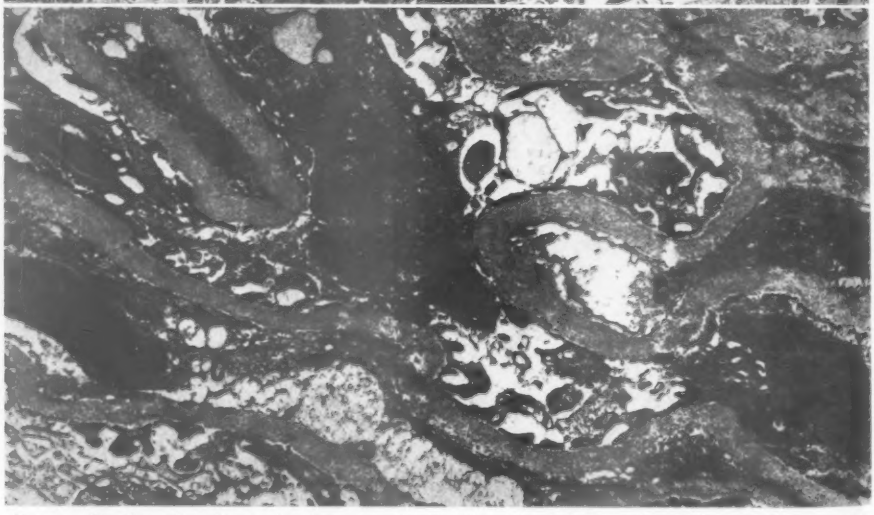


FIG. 4. Portion of a glomerulus from a patient with orthostatic proteinuria (case 1). In areas indicated by "X" the glomerular capillaries are collapsed and adherent, and endothelial cells (END) are somewhat swollen. × 3,000.

FIG. 5. A glomerulus in case 2 with orthostatic proteinuria. The glomerular capillaries (C) are narrow or obliterated, and the endothelial cells are swollen. There is an electron-dense deposit (D) in the cytoplasm of a glomerular epithelial cell. × 10,500.

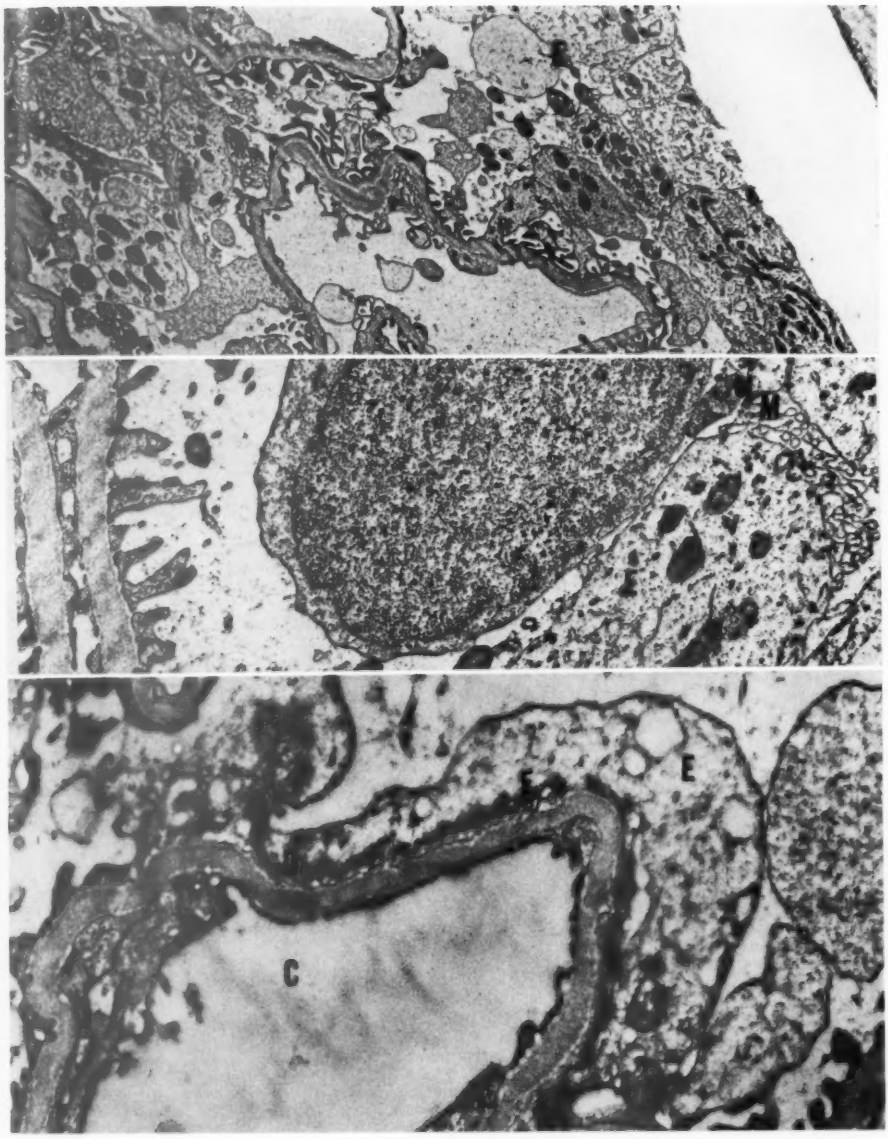


FIG. 6. A glomerulus in case 3. The glomerular epithelum (E) is swollen and its cytoplasm is "watery" in appearance. × 4200.

FIG. 7. A glomerulus in case 4. The cytoplasm in the glomerular epithelium (E) is swollen, and villiform membrane foldings (M) are present in one area. × 10,000.

FIG. 8. A glomerular capillary loop (C) in case 3. The foot processes (F) are fused over a portion of the loop. A zone of density (D) remains in the area originally occupied by the foot processes. The epithelial cytoplasm (E) over the area of fused foot processes is swollen and "watery." × 14,500.

FIG. 9. Foot process (P) fusion occurs over two capillary loops. At "X" minute notches remain, indicating sites where the interpedicel spaces have been "ironed out" and almost obliterated. × 18,000.

FIG. 10. A portion of glomerulus in case 2. The epithelial cytoplasm (E) is swollen and contains many dense, finely granular deposits (D), × 14,500.

FIG. 11. Portion of a glomerular epithelial cell (E) containing discrete, dense bodies (X) in the cytoplasm. These are comparable in size and location to PAS-positive hyaline droplets observed with light microscopy. Pinocytotic vesicles (P) are also present. × 18,000.

FIG. 12. Portion of a glomerular basement membrane (B) in case 4. A fine fibrillar structure is apparent. At "X" fine strandlike deposits of basement membrane substance are seen between the endothelial lining (END) and the basement membrane proper. Portions of pedicels of epithelial cells (E) appear on the outer surface of the basement membrane. × 47,000.

FIG. 13. Portion of a glomerular capillary (C) in case 4. The basement membrane (B) is "split" or laminated. An area of fused epithelial foot processes (F) can be seen, while at "X" the pedicels are still intact. × 14,500.

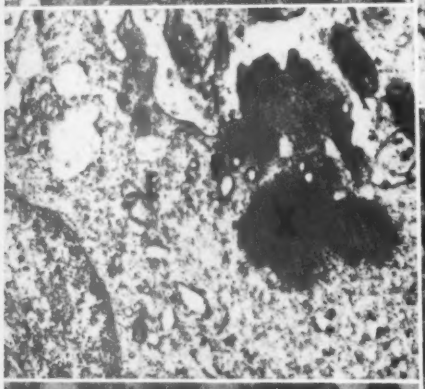
9

11

12

9

10



11

12

13

STUDIES ON THE MECHANISM OF ALTERED SUSCEPTIBILITY TO EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

SEYMOUR LEVINE, M.D., AND EUGENE J. WENK, M.A.

From the Department of Pathology, St. Francis Hospital, Jersey City, N.J.

Experimental allergic encephalomyelitis (EAE) (Fig. 1) was induced in the rat by Lipton and Freund in 1953.[1] They succeeded in a species previously thought to be resistant because they administered the encephalitogenic emulsion of nervous tissue plus adjuvant by the intracutaneous route. Thereafter, the rat was used for numerous EAE experiments: passive transfer by parabiosis[2] and by lymph node cells,[3,4] immunologic tolerance[5] or unresponsiveness,[6] induced localization,[7–9] and others. The present study concerns factors that influence the incidence and severity of EAE in rats: strain of origin, composition of adjuvant, route of administration, and prior pertussis vaccination. The results point to a common mechanism for the increased susceptibility to EAE in our experiments, namely, augmentation of inflammatory response to encephalitogenic emulsion.

METHODS

Female rats, usually 200 to 250 gm., of the following strains were used: Long-Evans hooded (Rockland Farms), Sherman (Rockland Farms), CFN (Carworth Farms), CFN-derived (derived from Carworth Farms CFN but obtained from another source), HH Wistar (Hemlock Hollow Farms), and Sprague-Dawley (Sprague-Dawley, Inc.).

The antigen introduced was a 33 per cent homogenate of previously frozen rat, guinea pig or bovine spinal cord in distilled water (with phenol in final concentration of 0.5 per cent). This was prepared in a Waring Blender or by cycling between two syringes connected by an 18-gauge emulsion needle or ordinary 18-gauge needles joined by PE 190 polyethylene tubing (Chase emulsifier). The homogenate was emulsified in an equal volume of adjuvant in the emulsifier.

Complete adjuvant contained 8.5 parts Bayol F, 1.5 parts Arlacel A, and heat-killed, dry human tubercle bacilli, usually 4 mg. per ml., but in some experiments 20 or 140 mg. per ml. Incomplete adjuvant was the same except for the omission of tubercle bacilli.

Under ether anesthesia, the emulsion was injected in 7 intradermal sites, 0.1 ml. each, on the flanks and neck, or in a single foot pad with an injection of 0.05 ml. Purina Laboratory Chow and tap water were fed *ad libitum*. In some experiments (numbers 3, 4 and 8), paralyzed rats were segregated, hand-fed, and bladder urine expressed manually as needed to promote survival; in other experiments, rats were killed within a few days of the onset of symptoms. All survivors were sacrificed 27 or 28 days after challenge by exsanguination under ether or chloroform anesthesia.

This investigation was supported wholly by Grant No. 184-3 from the National Multiple Sclerosis Society.

Accepted for publication, May 19, 1961.

Tissues were fixed in buffered 10 per cent formalin. Skulls and vertebral columns were subsequently decalcified in 5 per cent nitric acid for later exposure of the middle ears and spinal cords. Spleens were weighed after fixation. Tissues were embedded in paraffin, sectioned, and stained by the hematoxylin and eosin, Ziehl-Neelsen, and the Luxol fast blue-periodic acid-Schiff-hematoxylin stains. The tissues examined included central nervous system, lung, spleen, liver, kidney, injection sites, and lymph nodes. Sections were examined without knowledge of the experimental group from which the sample was derived.

The entire brain (except the olfactory lobes) in coronal slices and the spinal cord in longitudinal section were studied in 260 of the rats used in these experiments. EAE was present in 166 animals, with involvement of cord in 155, hindbrain in 138, and forebrain in 112 instances. In all but one rat with EAE in more than one of these locations, the lesions were more severe and numerous in the spinal cord and hindbrain than in the forebrain. In addition, the cord was the sole site of lesions in 21, the hindbrain in 6, and the forebrain in only 4 cases (the last were all minimal lesions). None of the variables employed in the experiments influenced this pattern of topographic distribution. Therefore, study of the forebrain was omitted in subsequent experiments.

Scoring of the intensity of EAE was modified after Alvord and Kies [10] and Waksman.[11] The clinical score included a grade of 1 to 4 based on the degrees of weakness, number of limbs affected and the presence of urinary incontinence, plus an additional grade of 1 to 4 based on whether clinical signs had their onset 17 to 21, 14 to 16, 12 to 13, or 9 to 11 days after inoculation. The highest possible clinical score was 8. The histologic grade of 1 to 4 was based on the number and severity of lesions (Fig. 1). In order to give histologic and clinical data equal weight, the histologic grade from the most severely involved part of the nervous system (usually the cord or hindbrain) was doubled before being added to the clinical score to give the final score.

Inoculation sites were scored clinically in arbitrary grades of 1 to 4, based on ulceration, crusting and the size of lesions; histologic grades of 1 to 4 were based on the extent of ulceration and suppuration (Figs. 2 to 5). The extent of metastatic spread of encephalitogenic emulsion was evaluated by scoring pertinent lesions in the lymph nodes, lung and spleen. Lymph nodes contained granulomas which were too numerous and confluent to count; therefore, lymph node scores were based on the number and size of all foci of necrosis and suppuration (Figs. 6 and 7). Lung and spleen, embedded in their entirety, were scored by counts of granulomas (Figs. 8 and 9) detected at a magnification of 35 times. In one of the experiments (number 11), lung granulomas were too numerous to count; therefore, stained sections were viewed macroscopically, and lung scores were based on the number of miliary tubercle-like foci detected with the unaided eye.

Results

Strain Differences [12–14]

The susceptibilities to EAE in 5 rat strains were determined in experiments 1, 2, 3 and 4 (Table I). Each rat was challenged with 120 mg. of rat spinal cord antigen emulsified with an adjuvant containing 1.4 mg. of tubercle bacilli; this was distributed among 7 intradermal sites on the flanks and neck.[1] Sherman albino rats were more susceptible to EAE than Long-Evans hooded animals although both were purchased from the same source and were raised under allegedly identical conditions. HH Wistar rats were far more susceptible than CFN or Sprague-Dawley rats. More susceptible strains showed earlier onset, higher incidence and

greater severity of the clinical signs and histologic lesions of EAE, but only final scores are recorded in Table I.

Sites of inoculation showed progressive enlargement, inflammation, ulceration and crusting, followed by healing. Histologically, the skin showed epithelioid cell granulomas, lymphocytes and plasma cells, focal suppuration and ulceration (Figs. 2 to 5). There were round spaces containing acid-fast bacilli which represented residual droplets of inoculum (Figs. 4 and 5). There was variation among the several sites in a single rat and among the several rats in a single strain, but the average reactivity for a given strain usually paralleled susceptibility to EAE (Table I and Figs. 2 to 5). Inoculation sites in the more susceptible HH Wistar rats showed a longer period of progressive enlargement, larger size, more ulceration and less healing than those in less susceptible Sprague-Dawley rats; the average clinical grades at sacrifice were 2 and 1.4 respectively. Histologically, HH Wistar, Sherman and Long-Evans rats showed a higher incidence and a greater severity of suppuration and ulceration and somewhat more extensive granulomatous inflammation than CFN rats, while Sprague-Dawley rats showed the least amount of reaction.

Inguinal, elbow, axillary and cervical lymph nodes were enlarged and contained noncaseating, frequently confluent epithelioid cell granulomas, most numerous under the capsule. There were clear round spaces containing acid-fast bacilli (due to metastatic spread of the oily inoculum[15]), often with focal necrosis and suppuration and perifocal fibrosis and chronic inflammation (Fig. 7). Suppurative foci (Fig. 6) were numerous and large in almost all highly susceptible HH Wistar rats but were few or absent in the less susceptible Sprague-Dawley rats.

Lungs contained scattered or confluent noncaseating granulomas,[15] frequently subpleural, less often in the deep parenchyma. Langhans' giant cells occurred alone or in granulomas (Fig. 8). Pulmonary granulomas were much more numerous among HH Wistar rats than among Sprague-Dawley rats. There was correlation between the lymph node scores and the lung granuloma counts for individual rats. This suggested that inoculum particles which reached the blood stream (and hence the lungs) had come via the lymphatic system. Sherman and Long-Evans rats had nearly equal lung granuloma counts despite differences in susceptibility to EAE; as noted above, both strains showed strongly reactive inoculation sites.

Splenic granulomas(Fig. 9) occurred in small numbers in HH Wistar, Long-Evans and Sherman strains only. However, this finding is of doubtful significance because sections of entire spleens from 5 normal untreated rats in each strain showed 7 to 16 granulomas in Long-Evans and

TABLE I

EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS (EAE) IN 5 STRAINS OF RATS *

Exper. no.	No. of rats	Strain	EAE score	Injection site score †	Lymph node score	Lung granulomas	Spleen wt. (%)	Otitis media
1	10	Sherman	7.4	2.7			.41	8/9
	8	Long-Evans	2.3	3.1			.31	6/8
	10	HH Wistar	5.9	3.5			.40	3/9
	9	Sprague-Dawley	1.4	0.4			.20	0/6
	11	CFN	2.9	1.5			.21	1/10
2	13	Sherman	5.9			8	.42	11/13
	14	Long-Evans	2.4			12	.38	14/14
3	10	HH Wistar	11.5	2.0	20.0	32	.35	3/9
	9	Sprague-Dawley	2.9	1.4	1.1	3	.24	0/9
4	20	Sprague-Dawley	2.6	1.4	0.3		.21	1/18

* Rats challenged with 120 mg. of rat cord and complete adjuvant containing 1.4 mg. of killed tubercle bacilli, distributed among 7 intradermal sites. In this and subsequent tables, scores are group averages and spleen weights are expressed as average per cent of body weight at sacrifice. Otitis media recorded as number positive (unilateral or bilateral) over total number examined.

† Histologic scores (see text for explanation) in experiment 1: clinical scores at time of sacrifice in experiments 3 and 4.

5 to 23 granulomas in Sherman rats. Spleen weights in the Sprague-Dawley and CFN strains were consistently lower than the others in experimental and in normal untreated groups. Histologically, small spleens had less red pulp than large spleens.

Differences in spleen size and histologic structure might indicate differences in past or present, latent or active infection. The incidence of chronic suppurative otitis media did, indeed, parallel spleen size and was low only in Sprague-Dawley and CFN rats both in experimental and in normal untreated groups. (However, there was poor correlation between otitis and large spleens for individual rats.) Furthermore, lungs of experimental and untreated Sprague-Dawley and CFN rats had less peribronchial and perivascular lymphoid tissue than other strains. Lungs of some "normal" rats, especially HH Wistar and Sherman, contained foci of bronchopneumonia, interstitial mononuclear inflammatory cells, intra-alveolar foamy macrophages, and even occasional epithelioid cell granulomas.

In summary, comparison of HH Wistar and Sherman with Sprague-Dawley and CFN rats showed that the two former strains were more susceptible to EAE and exhibited greater inflammatory reaction to the encephalitogenic inoculum, more extensive dissemination of granulomas in lymph nodes and lungs, larger spleens, more lymphoid tissue and greater incidence of inflammation in lungs and middle ears. However, these correlations were imperfect in explaining results with individual rats; and the Long-Evans strain was anomalous in showing low susceptibility to EAE despite ample inflammatory reaction, fairly large spleens, and high incidence of otitis media.

Pretreatment with Pertussis Vaccine in Rat Strains of Low Susceptibility

Lee and Olitsky [16] showed that inoculation of BSVS mice with *Hemophilis pertussis* vaccine increased susceptibility to EAE. Similar results were obtained in 2 rat strains of low reactivity in experiments 4, 5 and 6 (Table II). Pertussis vaccine, supplied by Dr. John A. McMillen of Lederle Laboratories (phase I, 0.6 ml., containing approximately 36 billion organisms, diluted to 3 ml. with sterile saline) was inoculated intraperitoneally 4 days before challenge. Encephalitogenic emulsion was injected intradermally as in the preceding experiments. The pretreatment produced a marked increase in the incidence and severity of EAE. Multiple pertussis injections had an even greater effect. Outdated vaccine was partly effective. Control rats pretreated with saline did not differ significantly from controls that had no injection before challenge. Omission of tubercle bacilli from the adjuvant ("incomplete adjuvant") rendered the encephalitogenic emulsion ineffective in a strain of low susceptibility, even after pertussis vaccination.

TABLE II

ENHANCEMENT OF EAE BY PERTUSSIS VACCINATION *

Exper. no.	No. of rats	Strain	Pretreatment	Adjuvant	EAE score	Injection site score †	Lymph node score	Lung granulomas	Spleen wt. (%)
4	20	Sprague-Dawley	None	Complete	2.6	1.4	0.3		.21
	10	Sprague-Dawley	Pertussis	Complete	10.7	2.1	3.0		.43
5	20	CFN-derived	None	Complete	0.7				.19
	20	CFN-derived	Saline	Complete	1.3				.18
	20	CFN-derived	Pertussis	Complete	3.5				.24
6	10	CFN-derived	Saline	Complete	0.5	0.4	0.0	1.7	.19
	10	CFN-derived	Pertussis	Complete	6.5	2.5	3.1	14.6	.27
	10	CFN-derived	Pertussis ‡	Complete	10.1	3.3	4.1	15.0	.37
	9	CFN-derived	Saline	Incomplete	0.0	0.0	0.0	0.0	.18
	10	CFN-derived	Pertussis	Incomplete	0.0	0.0	0.0	0.1	.27
7	5	HH Wistar	Saline	Complete	6.2				
	8	HH Wistar	Pertussis §	Complete	8.5				

* Rats challenged with 120 mg. of rat cord (except guinea pig cord, experiment 7) and complete adjuvant containing 1.4 mg. of killed tubercle bacilli (or incomplete adjuvant, i.e., tubercle bacilli omitted), distributed among 7 intradermal sites. Pertussis vaccine, 0.6 ml. diluted to 3 ml. with saline, or plain sterile saline, 3 ml., injected in peritoneal cavity 4 days before challenge. Outdated vaccine used for experiment 5.

† Histologic scores, experiment 6; clinical scores, experiment 4.

‡ Pertussis vaccine twice weekly starting 4 days before challenge.

§ Pertussis vaccine, 1 ml., undiluted, 4 days before challenge.

Pertussis pretreatment increased inflammation at the sites of inoculation of encephalitogenic emulsion containing complete adjuvant; average clinical scores at time of sacrifice were 2.1 (range, 1.8 to 2.6) compared to 1.4 (range, 1 to 1.9) for controls. Histologic scores showed a comparable effect and even more after multiple pertussis injections. Pertussis pretreatment had no effect on the sites of inoculation when an incomplete adjuvant was utilized; these sites had minimal granulomatous response and no suppuration or ulceration (Fig. 11).

Lymph nodes (cervical, elbow, axillary, and inguinal) showed analogous differences. All rats receiving encephalitogenic emulsion with complete adjuvant showed at least a few granulomas in lymph nodes, but these were far more numerous in the pertussis-pretreated groups. In one experiment, foci of suppuration and necrosis around oil droplets were evident in lymph nodes in 8 of 10 in the pertussis group (maximum score, 16; average, 3), but only in 5 of 20 controls (maximum score, 2.5; average, 0.3). When the encephalitogenic emulsion was made with the incomplete adjuvant (oil but no tubercle bacilli), the lymph nodes contained oil droplets but not granulomas (Fig. 13).

Lung granulomas were more frequent in pertussis-pretreated rats (incidence, 7 of 10; average number, 14.6) than in controls (incidence, 5 of 10; average number, 1.7). Only one granuloma was found in a single rat when the adjuvant lacked tubercle bacilli.

Spleen weight was increased by intraperitoneal pertussis vaccination.[17–19] Examination of entire spleens in pertussis-treated rats showed epithelioid cell granulomas in most cases, varying from a few to hundreds, usually in the red pulp. Necrosis, suppuration, oil droplets, and acid-fast bacilli were absent. Splenic granulomas occurred in all pertussis-treated groups, even those in which encephalitogenic emulsion lacked tubercle bacilli, or (in other experiments not described here) in which no encephalitogenic emulsion was administered. They did not occur in the absence of pertussis treatment in these strains of rats even though the encephalitogenic emulsion contained tubercle bacilli. Therefore, they were not due to metastasis of particles of encephalitogenic emulsion as in the lungs and superficial lymph nodes which drain the skin inoculation sites. Mediastinal and perirenal lymph nodes draining the peritoneal cavity were enlarged and contained granulomas (as in the spleen) only in pertussis-treated rats. Mesenteric lymph nodes, draining neither peritoneal cavity nor skin inoculation sites, showed no granulomas in any group. Granulomas in the liver paralleled those observed in the spleen. Multiple pertussis injections produced adrenal enlargement and thymic atrophy, suggesting a "stress" effect.

Pretreatment with Pertussis Vaccine in a Rat Strain of High Susceptibility

It was of interest to extend the study of pertussis effects to HH Wistar rats, a strain of high reactivity. In experiment 7 (Table II) all control as well as pertussis-pretreated HH Wistar rats developed EAE following intradermal inoculation of guinea pig cord emulsion in complete adjuvant. The average scores suggested that pertussis pretreatment had accelerated and intensified EAE, but the high degree of reactivity in both groups made this small difference of uncertain significance.

A further approach was made possible by the observation of Bell and Paterson[20] that EAE could be induced in rats even though acid-fast bacilli were omitted from the adjuvant. In a preceding experiment we had failed to produce EAE in a strain of low susceptibility treated with emulsion containing incomplete adjuvant, but we confirmed Bell and Paterson's observations when we used highly reactive HH Wistar rats. In experiment 8 (Table III) the effect of intraperitoneal pertussis vaccination 4 days before challenge was evaluated. Guinea pig cord antigen with incomplete adjuvant proved so potent that the incidence of EAE was equally high in control and pertussis-pretreated rats, but the latter had slightly higher scores.

TABLE III

EFFECT OF PERTUSSIS ON EAE INDUCED WITH INCOMPLETE ADJUVANT *

Exper. no.	No. of rats	Type of cord antigen	Pretreatment	EAE score	Spleen wt. (%)
8	18	Guinea pig	Saline/nothing	6.7	.41
	16	Guinea pig	Pertussis	7.7	.46
9	9	Guinea pig	Saline/nothing	2.8	.41
	4	Guinea pig	Pertussis	4.0	.73
	9	Rat	Saline/nothing	0.3	.39
	6	Rat	Pertussis	4.5	.56
	10	Bovine	Saline/nothing	0.5	.37
	4	Bovine	Pertussis	6.5	.53

* HH Wistar rats challenged with 120 mg. of spinal cord antigen and incomplete adjuvant, distributed among 7 intradermal sites. Pertussis vaccine given intraperitoneally 4 days before challenge in both experiments, repeated twice weekly for 4 additional doses in experiment 9. Half of controls untreated, half received equal volume of saline.

In experiment 9 (Table III) incomplete adjuvant with rat, bovine, or guinea pig cord antigens were tested. Pertussis was administered 4 days before challenge, and repeated on the day of challenge and 3 times during the next 10 days. Thereafter, injections were discontinued because

severe ascites developed.[21] With guinea pig cord antigen, pertussis-treated rats had, again, only slightly higher scores than controls without pertussis. Rat cord and bovine cord were less potent antigens, and the incidence of EAE in controls was low. Under these conditions, pertussis treatment produced definite enhancement of EAE.

The sites of inoculation of encephalitogenic emulsion with incomplete adjuvant showed moderate granulomatous inflammation (Fig. 10) with occasional ulceration. This was in contrast to the dearth of inflammatory cells (Fig. 11) found in a strain of low susceptibility in a preceding experiment. Pertussis pretreatment increased the incidence and severity of ulceration. Lymph nodes in these HH Wistar rats contained no suppurative foci but did exhibit oil droplets with epithelioid cell granulomas (Fig. 12); lymph nodes in the poorly reactive strain in a preceding experiment showed oil droplets with little or no cellular reaction (Fig. 13). Although spleens in HH Wistar rats were normally large, single pertussis injections produced a slight, and multiple pertussis injections a further increase in size, and there were epithelioid cell granulomas.

In summary, pertussis vaccination increased susceptibility to EAE in rats and augmented the inflammatory reaction at inoculation sites and the number of lesions in draining superficial lymph nodes and the lungs. The enhancing effect of pertussis pretreatment was greatest in strains of low reactivity but could be demonstrated in strongly reactive strains, especially when relatively ineffective antigen and adjuvant were deliberately chosen so as to lower the degree of susceptibility.

Composition of Adjuvant

Although highly susceptible HH Wistar rats developed EAE whether or not the adjuvant contained tubercle bacilli, less reactive strains developed EAE only when complete adjuvant was employed. Even with complete adjuvant the incidence of EAE in CFN-derived rats was low, but this was increased in experiment 10 by fortification of the adjuvant with increased amounts of tubercle bacilli. Rats were challenged with 120 mg. of rat or bovine cord in adjuvant containing 1.4 mg. (amount in previous experiments), 7 mg., or 49 mg. of killed tubercle bacilli, divided among 7 intradermal sites. The incidence and severity of EAE increased with larger amounts of tubercle bacilli (Table IV).

The sites of inoculation with low dosage material showed little ulceration, suppuration, or granulomatous response; this was similar to the observations among CFN and Sprague-Dawley rats at the same dosage level in experiments 1 and 3. Higher dosage groups showed more ulceration, suppuration and granulomas. Lesions in lymph nodes and lungs

TABLE IV

EFFECT OF COMPOSITION OF ADJUVANT ON EAE *

Exper. no.	No. of rats	Type of antigen	Tubercle bacilli in adjuvant	EAE score	Injection site score †	Lung granulomas	Spleen granulomas	Spleen wt. (%)
10	10	Rat cord	1.4 mg.	1.9	0.9	1.4	0.0	.21
	10	Rat cord	7.0 mg.	5.2	2.4	14.0	1.4	.21
	10	Rat cord	49.0 mg.	6.8	3.8	47.0	62.0	.30
	10	Bovine cord	7.0 mg.	3.5			0.7	.21

* CFN-derived rats challenged with 120 mg. of spinal cord antigen and complete adjuvant with indicated amounts of tubercle bacilli, distributed among 7 intradermal sites.

† Histologic scores.

TABLE V

COMPOSITION OF ADJUVANT, PERTUSSIS PRETREATMENT, ROUTE OF INOCULATION, AND ADDITIVE EFFECTS

Exper. no.	No. of rats	Strain	Pretreatment	Tubercle bacilli in adjuvant	Route *	EAE score	Lymph node score	Lung granulomas †	Spleen wt. (%)
11	10	CFN-derived	Saline	7.0 mg.	ID	3.4	5.3	1.1	.20
	10	CFN-derived	Pertussis	7.0 mg.	ID	9.4	13.2	22.5	.35
	10	CFN-derived	Saline	49.0 mg.	ID	0.8	17.8	34.2	.26
	9	CFN-derived	Pertussis	49.0 mg.	ID	3.2	26.2	29.3	.40
3	9	HH Wistar	None	0.1 mg.	FP	13.9	4.7 ‡		
	10	HH Wistar	None	1.4 mg.	ID	11.5	20.0	32.0	.35
	9	Sprague-Dawley	None	0.1 mg.	FP	13.6	2.0 ‡		
	9	Sprague-Dawley	None	1.4 mg.	ID	2.9	1.1	3.0	.24
12	5	HH Wistar	Saline	1.4 mg.	SC	0.0	0.2		.34
	5	HH Wistar	Pertussis	1.4 mg.	SC	3.2	0.4		.55
	5	HH Wistar	Saline	7.0 mg.	SC	3.0	2.0	19.0	.39
	5	HH Wistar	Pertussis	7.0 mg.	SC	8.6	2.4	16.0	.39

* ID = intradermal challenge with 120 mg. of rat cord and adjuvant as indicated, 0.1 ml. in each of 7 sites. SC = subcutaneous challenge as above. FP = foot pad challenge with 8.6 mg. of rat cord and adjuvant as indicated, 0.05 ml. in a single site. Pretreatments given intraperitoneally 4 days before challenge.

† Figures for experiment 11 are average scores obtained by counting macroscopically visible conglomerates of granulomas in stained sections of entire lungs; all other lung granuloma figures represent microscopic counts.

‡ All the suppurative foci around oil droplets represented by these figures were found in the homolateral popliteal (Sprague-Dawley) or popliteal and inguinal (HH Wistar) lymph nodes. Only scattered granulomas were detected in other nodes.

were rare in the low dosage group and more numerous with the higher dosages. Acid-fast bacilli, oil droplets and suppuration were common in lymph node and even in lung lesions of high dosage groups (Fig. 14). Splenic granulomas (Fig. 9) were absent with the low dosage, present in 2 rats in the middle dosage group, and in 8 rats in the highest dosage group, often in large numbers. Rare acid-fast bacilli were found in splenic granulomas, indicating that in this instance granulomas were due to metastatic spread of the emulsion through the pulmonary filter. The numbers of lesions in lymph nodes, lung and spleen were fairly well correlated for individual rats. Granulomas occurred in the liver as well.

Additive Effects of Pertussis and Adjuvant Fortification

In experiment 11 (Table V), both pertussis pretreatment and adjuvant fortification were administered simultaneously to a strain with low susceptibility. Seven mg. of tubercle bacilli induced a high incidence of EAE, but the addition of pertussis pretreatment gave a still greater effect, with 100 per cent incidence of clinically apparent EAE (Table V). Interestingly, increase of tubercle bacillus dosage to 49 mg. caused a reduction of EAE, unlike the findings in the previous experiment, but corresponding to observations in guinea pigs given doses above optimum.[22] Even at this supra-optimal level, pertussis pretreatment enhanced EAE. The large amounts of adjuvant used in this experiment caused polyarthritis,[23] especially at the 49 mg. dose level and after pertussis pretreatment.

As in the previous experiment, large doses of tubercle bacilli caused severe inflammation and ulceration at inoculation sites, numerous granulomas and suppurative foci in the lymph nodes and lungs. Pertussis pretreatment enhanced the severity and the numbers of lesions in the skin, nodes and lungs, especially at the 7 mg. tubercle bacillus dose level (Table V). Spleens were increased in weight and contained granulomas, in this instance attributable both to circulating particles of adjuvant and to pertussis treatment.

Route of Inoculation of Encephalitogenic Emulsion

Waksman, Pearson and Sharp[23] have reported the greater efficacy of the foot pad route as compared to intradermal flank inoculations of encephalitogenic emulsions; this was confirmed in experiment 3 (Table V). In the rat strain with low susceptibility (Sprague-Dawley), the injection of emulsion (0.05 ml.) in one foot pad was much more effective than the injection of 0.1 ml. in each of 7 intradermal sites on the flank and neck. The resulting incidence and severity of EAE could be achieved in this strain by intradermal inoculation only with the aid of pertussis

pretreatment or fortification of the adjuvant. In highly susceptible HH Wistar rats, either inoculation route gave good results, but foot pad inoculation yielded higher EAE scores, attributable to an earlier onset.

Local inflammation was much more severe and prolonged in the foot pad than in the flank or neck sites. Histologically, oil droplets were found to have spread widely. Granulomatous and suppurative inflammation involved the skin, muscle, nerve, tendon, tendon sheath, joint and bone; there was also subperiosteal new-bone deposition. Granulomatous lymphangitis extended from the foot to the leg. Both foot pad and flank-neck sites exhibited more severe lesions in the HH Wistar than in the Sprague-Dawley rats. Granulomas were present in all homolateral popliteal, most homolateral inguinal and some lower lumbar lymph nodes, less commonly in other nodes, following foot pad inoculation (Fig. 15). Suppurative foci about oil drops were more frequent and larger in the popliteal nodes of the HH Wistar than in the Sprague-Dawley rats. Lesions were present in some inguinal nodes only in the HH Wistar rats. It is of interest that the single popliteal node of Sprague-Dawley rats contained as many suppurative foci following foot pad inoculation as did all the axillary, elbow, inguinal and cervical nodes after flank-neck inoculations, although the doses of injected emulsion were in the ratio of 1 to 14.

In contrast to the foot pad and intradermal routes, the subcutaneous route is considered ineffective for producing EAE in rats.[1,24] In experiment 12 (Table V) we found that subcutaneous inoculation with 0.7 ml. of encephalitogenic emulsion containing 1.4 mg. of tubercle bacilli, when divided equally among 7 sites on the flanks and neck, failed to elicit EAE even in highly susceptible HH Wistar rats. Pertussis pretreatment or fortification of the adjuvant (7 mg. of tubercle bacilli) induced EAE in a few rats, but better results were obtained by utilizing both ancillary measures together.

The subcutaneous inoculation sites contained less granulomatous inflammation than the intradermal sites. Frequently, the oily inoculum was present in a single cyst several millimeters in diameter with little surrounding reaction. The entire lesion was often encapsulated but occasionally it was adherent to the skin, with involvement of the panniculus carnosus and dermis; occasionally there was ulceration. Lymph nodes showed very few suppurative foci around oil drops; even rats that received pertussis and high dosages of tubercle bacilli showed few such foci.

Discussion

Differences in susceptibility to EAE among rat strains, as demonstrated here, are analogous to differences among strains of guinea pigs,[12]

mice,[13] and dogs,[14] noted by others, and help to explain the varying success which different investigators have experienced in the rat.[4] Our observations on spleen size and histologic structure, pulmonary alterations, otitis media, the incidence of EAE, and the degree of local inflammatory reaction probably represent only a fraction of the intrinsic differences among these strains. Strain variations might reflect genetic background or environment prior to arrival in our laboratory, or additive or interacting combinations. Large spleens suggest exposure to pathogenic micro-organisms; we found no Salmonella in cultures of cecal contents from the HH Wistar rats, but these rats are known to carry latent infection with *Hemobartonella muris*.[3] It is of interest that spleen weights in our CFN, CFN-derived and Sprague-Dawley rats were close to those reported by Gordon and Wostmann[25] for "germfree" rats, while spleen weights in our HH Wistar, Sherman and Long-Evans strains were close to the values given for "conventional" rats. Otitis media probably has no direct relation to susceptibility to EAE; it is one of numerous sources of exposure to micro-organisms and to products of leukocytic disintegration, the sum total of which may contribute to susceptibility, and perhaps also to splenic enlargement.

As to the mechanism by which strain differences operate to determine susceptibility to EAE, we have demonstrated different degrees of inflammatory response to encephalitogenic emulsion; this is supported by parallel observations by Waksman in 2 rabbit strains[26] and by Paterson in hamsters.[27] However, imperfect correlations for individual rats and anomalous results with the Long-Evans strain indicate that the inflammatory response is not the sole determining factor for susceptibility to EAE. Also, we have shown that the amount of spread of encephalitogenic emulsion to lymph nodes and lungs is usually greater in rat strains of high EAE susceptibility than in those with low susceptibility. This may be significant because these foci are sources of antigenic stimulation[15]; in fact, the draining lymph node is essential in the development of EAE in rabbits.[28]

Pertussis vaccine pretreatment also increased inflammation at inoculation sites, in lesions in draining lymph nodes and in the lungs. However, leukocytosis,[17-19] permeability effects,[29] pyrogenicity, or histamine sensitization[30] may be related or additional factors in the pertussis effect. Splenic enlargement after the introduction of pertussis organisms and the "natural" occurrence of large spleens in susceptible strains may be more than coincidence. One might suppose that susceptible strains have a "built-in" pertussis effect.

The efficacy of foot pad, intradermal and subcutaneous routes has been correlated with the degree of inflammation noted in each site. The intense inflammation after foot pad inoculation may be due to the con-

tinual massage, trauma and even infection which this area sustains. The volume of emulsion administered in flank-neck intradermal injections was 14 times that given in the foot pad. The lesser effectiveness of multiple intradermal injections was not, however, attributable to overdosage; Waksman, Pearson and Sharp[23] found the foot pad to be a more effective site than the flank when equal volumes were injected.

We have correlated the incidence of EAE with the degree of inflammation at inoculation sites under several experimental circumstances: strain variation, pretreatment with pertussis organisms, changes in adjuvant composition, and the route of inoculation. To these may be added the experiments of Kabat, Wolf and Bezer with cortisone[31] and with adjuvants other than mycobacteria[32] in which the same correlation was shown. Taken together, these data indicate the fundamental role of the inflammatory response in the pathogenesis of EAE. We have used suppuration as a convenient index for scoring lesions in the skin and lymph nodes, but this does not imply any special role for neutrophils in the development of EAE. In fact, the emulsion prepared with an incomplete adjuvant produced no suppurative lesions in lymph nodes; susceptibility to its encephalitogenic activity was here correlated with epithelioid cell response (Figs. 12 and 13), in substantiation of the results obtained by Kabat, Wolf and Bezer.[31-33]

Increase in the size and number of lesions in lymph nodes and lungs have been noted under the same circumstances that have augmented inflammation at inoculation sites. This was due partly to heightened tissue reactivity in susceptible compared to resistant rat strains, and in pertussis-vaccinated compared to untreated rats. For example, droplets of emulsion with incomplete adjuvant failed to elicit inflammation in the lymph nodes of CFN-derived rats, while they excited a granulomatous response in the nodes of highly susceptible HH Wistar rats (Figs. 12 and 13). However, it seems unlikely that heightened tissue reactivity is the entire explanation, because the increased number of lymph node and lung lesions was often out of proportion to the increase in the intensity of the local skin inflammation. Moreover, this could not explain the high incidence of lymph node lesions after foot pad inoculation (Fig. 15), as there is little reason to suspect the popliteal node of greater reactivity than any other lymph node. We suggest that the severity of inflammation at local inoculation sites determines the extent to which tissue pressure increases and hence the amount of lymphatic transport of encephalitogenic emulsion and the number of lymph node and lung lesions. McMaster has shown that there is a slight gradient of pressure from tissue to lymph, which is increased during the development of inflammatory edema and is important in lymph formation.[34] Increased

tissue pressure may be responsible, also, for the ulceration of intradermal inoculation sites; this was extensive in susceptible strains (Fig. 2) and even in resistant strains after pertussis treatment, after injections with large doses of tubercle bacilli, and in foot pad sites. Increased tissue pressure is especially likely to develop after foot pad inoculation because of limited room for expansion in this region; indeed, the skin of an injected foot was obviously tense. The massage of continual movement and weight-bearing would further increase lymph flow. Intradermal injections, unlike subcutaneous ones, produce immediate entry of inoculum into lymphatics[35]; in addition, the subsequent development of an inflammatory lesion is more likely to be associated with persistently increased tissue pressure in the dense, firm dermis than in the soft subcutaneous fat. These considerations give a reasonable explanation for the association noted here between findings at inoculation sites and in draining lymph nodes.

Summary

A number of factors influencing the incidence and severity of experimental allergic encephalomyelitis (EAE) were subjected to experimental analysis. Various strains of rats exhibited differences in the incidence of EAE, usually correlated with the degree of inflammatory reaction at the site of inoculation of encephalitogenic emulsion and with the amount of metastatic extension of the emulsion to draining lymph nodes and lungs. Rat strains with the lowest susceptibility to EAE had certain other characteristics that set them apart: small spleens, low incidence of spontaneous otitis media, a paucity of peribronchial lymphoid tissue and inflammatory lesions in the lungs.

Pretreatment with pertussis vaccine increased the incidence and severity of EAE in strains with low susceptibility. Pertussis pretreatment also enhanced EAE in a strain of high susceptibility, especially when conditions were chosen to reduce the incidence of EAE (challenge by subcutaneous route or employment of incomplete adjuvant with certain antigens). Enhancement of EAE by pertussis vaccine was associated with increased inflammation in inoculation sites and increased number and size of lesions in the lymph nodes and lungs.

A single foot pad inoculation of encephalitogenic emulsion was more effective than multiple intradermal injections; the latter were more effective than multiple subcutaneous injections. The severity of local inflammation and of lesions in draining lymph nodes paralleled the intensity of EAE.

Homologous or heterologous spinal cord antigen induced EAE and provoked granulomatous inflammation in inoculation sites and lymph

nodes in a highly susceptible strain even though the adjuvant lacked tubercle bacilli. A strain with low susceptibility developed neither EAE nor granulomatous response under the same conditions. EAE was produced in the latter strain with a complete adjuvant containing killed tubercle bacilli and was enhanced by fortification of the adjuvant with increased amounts of tubercle bacilli. Simultaneous employment of high tubercle bacillus dosage and pertussis pretreatment produced additive enhancement, so that EAE could be induced even by the ordinarily ineffective subcutaneous route.

Susceptibility of rats to EAE was determined by the combined effects of inherent strain characteristics, pretreatment with pertussis vaccine, route of inoculation, and adjuvant composition. Increased inflammation at inoculation sites and the increased number and size of lesions in lymph nodes and lungs were the common accompaniments of all the experimental circumstances that resulted in a high incidence of EAE. This indicates the fundamental role of the inflammatory reaction in the pathogenesis of allergic encephalomyelitis.

References

1. Lipton, M. M., and Freund, J. Allergic encephalomyelitis in the rat induced by the intracutaneous injection of central nervous system tissue and adjuvants. *J. Immunol.*, 1953, **71**, 98–109.
2. Lipton, M. M., and Freund, J. The transfer of experimental allergic encephalomyelitis in the rat by means of parabiosis. *J. Immunol.*, 1953, **71**, 380–384.
3. Paterson, P. Y. Transfer of allergic encephalomyelitis in rats by means of lymph node cells. *J. Exper. Med.*, 1960, **111**, 119–136.
4. Koprowski, H.; Paraf, A.; Billingham, R., and Jervis, G. Études sur le mécanisme de l'encéphalite allergique chez le rat. *Compt. rend. Acad. sc.*, 1960, **250**, 2956–2958.
5. Paterson, P. Y. Studies of immunological tolerance to nervous tissue in rats. *Ann. New York Acad. Sc.*, 1958, **73**, 811–818.
6. Waksman, B. H. Allergic encephalomyelitis in rats and rabbits pretreated with nervous tissue. *J. Neuropath. & Exper. Neurol.*, 1959, **18**, 397–417.
7. Clark, G., and Bogdanove, L. H. The induction of the lesions of allergic meningoencephalomyelitis in a predetermined location. *J. Neuropath. & Exper. Neurol.*, 1955, **14**, 433–437.
8. Levine, S. Localization of allergic encephalomyelitis in lesions of cyanide encephalopathy. *J. Neuropath. & Exper. Neurol.*, 1960, **19**, 238–247.
9. Levine, S., and Wenk, E. J. Effect of induced localization on incidence and distribution of experimental allergic encephalomyelitis. *Neurology*, 1960, **10**, 1090–1095.
10. Alvord, E. C., Jr., and Kies, M. W. Clinico-pathologic correlations in experimental allergic encephalomyelitis. II. Development of an index for quantitative assay of encephalitogenic activity of "antigens." *J. Neuropath. & Exper. Neurol.*, 1959, **18**, 447–457.
11. Waksman, B. H.; Porter, H.; Lees, M. D.; Adams, R. D., and Folch, J. A study of the chemical nature of components of bovine white matter effective

in producing allergic encephalomyelitis in the rabbit. *J. Exper. Med.*, 1954, 100, 451–471.

12. LIPTON, M. M., and FREUND, J. The efficacy of the intracutaneous route of injection and the susceptibility of the Hartley strain of guinea pigs in experimental allergic encephalomyelitis. *J. Immunol.*, 1953, 70, 326–329.
13. OLITSKY, P. K., and LEE, J. M. Biological properties and variations of reactions of the encephalitogenic agent in nervous tissues. *J. Immunol.*, 1953, 71, 419–425.
14. JERVIS, G. A.; BURKHART, R. L., and KOPROWSKI, H. Demyelinating encephalomyelitis in the dog associated with antirabies vaccination. *Am. J. Hyg.*, 1949, 50, 14–26.
15. FREUND, J., and LIPTON, M. M. Experimental allergic encephalomyelitis after the excision of the injection site of antigen-adjuvant emulsion. *J. Immunol.*, 1955, 75, 454–459.
16. LEE, J. M., and OLITSKY, P. K. Simple method for enhancing development of acute disseminated encephalomyelitis in mice. *Proc. Soc. Exper. Biol. & Med.*, 1955, 89, 263–266.
17. LEE, J. M. Physio-pathological changes in mice following injection of *H. pertussis* vaccine. (Abstract) *Fed. Proc.*, 1959, 18, 579.
18. PARFENTJEV, I. A., and MANUELIDIS, E. E. Hemopoietic response in mice injected with pertussis vaccine. (Abstract) *Fed. Proc.*, 1956, 15, 607.
19. TUTA, J. A. A study of the lymphocytosis following intravenous injections into rabbits of suspensions and extracts of *Hemophilis pertussis*. *Folia haemat.*, 1937, 57, 122–135.
20. BELL, J., and PATERSON, P. Y. Rapid induction of allergic encephalomyelitis in rats without the use of mycobacteria. *Science*, 1960, 131, 1448.
21. LEVINE, S., and GRUENEWALD, R. Ascites in the rat produced by pertussis vaccine. (In press)
22. ALVORD, E. C., JR. Discussion in: "Allergic" Encephalomyelitis. Proceedings of a Symposium: Experimental "Allergic" Encephalomyelitis and Its Relation to Other Diseases of Man and Animals. Charles C Thomas, Springfield, 1959, p. 410.
23. WAKSMAN, B. H.; PEARSON, C. M., and SHARP, J. T. Studies of arthritis and other lesions induced in rats by injection of mycobacterial adjuvant. II. Evidence that the disease is a disseminated immunologic response to exogenous antigen. *J. Immunol.*, 1960, 85, 403–417.
24. LUMSDEN, C. E. Experimental "allergic" encephalomyelitis. *Brain*, 1949, 72, 198–226.
25. GORDON, H. A., and WOSTMANN, B. S. Morphological studies on the germfree albino rat. *Anat. Rec.*, 1960, 137, 65–70.
26. WAKSMAN, B. H. Discussion following KABAT, E. A.[28]
27. PATERSON, P. Y. Discussion in: "Allergic" Encephalomyelitis. Proceedings of a Symposium: Experimental "Allergic" Encephalomyelitis and Its Relation to Other Diseases of Man and Animals. Charles C Thomas, Springfield, 1959, p. 412.
28. CONDIE, R. M., and GOOD, R. A. Experimental Allergic Encephalomyelitis: Its Production, Prevention, and Pathology as Studied by Light and Electron Microscopy. In: Progress in Neurobiology. IV. The Biology of Myelin. KOREY, S. R. (ed.). Paul B. Hoeber, Inc., New York, 1959, pp. 321–392.
29. MUNOZ, J. Tissue permeability changes induced by *B. pertussis* in mice. (Abstract) *Fed. Proc.*, 1960, 19, Part 1, 246.

30. KIND, L. S. The altered reactivity of mice after inoculation with *Bordetella pertussis* vaccine. *Bact. Rev.*, 1958, **22**, 173–182.

31. KABAT, E. A.; WOLF, A., and BEZER, A. E. Studies on acute disseminated encephalomyelitis produced experimentally in rhesus monkeys. VII. The effect of cortisone. *J. Immunol.*, 1952, **68**, 265–275.

32. KABAT, E. A. Immunochemical Mechanisms in Experimental Acute Disseminated Encephalomyelitis and Their Relation to Multiple Sclerosis. Sixth International Congress of Neurology, Brussels, 1957, Reports and Discussions. Acta medica belgica, pp. 31–47.

33. KABAT, E. A.; WOLF, A., and BEZER, A. E. Studies on acute disseminated encephalomyelitis produced experimentally in rhesus monkeys. III. *J. Exper. Med.*, 1948, **88**, 417–426.

34. MCMASTER, P. D. The relative pressures within cutaneous lymphatic capillaries and the tissues. *J. Exper. Med.*, 1947, **86**, 293–308.

35. MCMASTER, P. D., and HUDACK, S. S. The formation of agglutinins within lymph nodes. *J. Exper. Med.*, 1935, **61**, 783–805.

[Illustrations follow]

Legends for Figures

Except where indicated, photomicrographs were prepared from sections stained with hematoxylin and eosin.

Fig. 1. Cerebellar white matter in a rat with EAE. Perivascular infiltration and demyelination are evident. Luxol fast blue-periodic acid-Schiff-hematoxylin stain. × 125.

Figures 2 to 9 show lesions in the skin, draining lymph nodes, lungs and spleen following intradermal inoculation of encephalitogenic emulsion containing complete adjuvant (i.e., killed tubercle bacilli).

Fig. 2. A rat from a strain with high susceptibility to EAE. There is ulceration, crusting, granulomatous inflammation, and focal suppuration in the inoculation site. × 125.

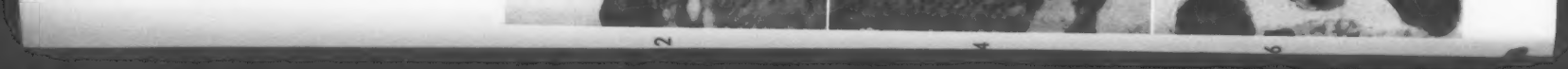
Fig. 3. A rat from a strain with low susceptibility to EAE. Granulomatous inflammation is manifest, but neither ulceration nor suppuration appear at the inoculation site. × 30.

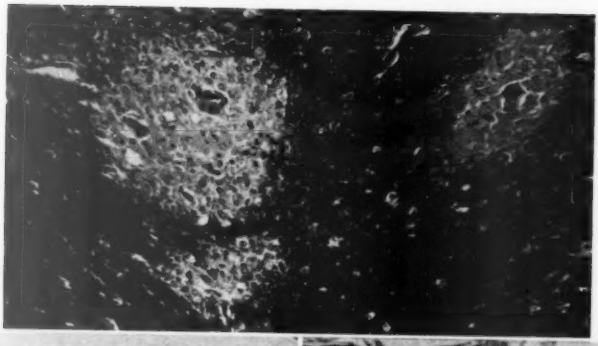
Fig. 4. The same lesion shown in Figure 2. Focal suppuration is demonstrated. The round spaces represent droplets of emulsion containing many acid-fast bacilli. × 125.

Fig. 5. A higher magnification of the lesion seen in Figure 3. There are emulsion droplets and granulomatous inflammation but no suppuration. × 125.

Fig. 6. A rat from a strain with high susceptibility to EAE. Multiple large, punched-out foci of necrosis and suppuration appear in inguinal, axillary, elbow and cervical lymph nodes (rat strains of low susceptibility showed few such foci unless given large amounts of tubercle bacilli or pretreated with pertussis vaccine). × 3.

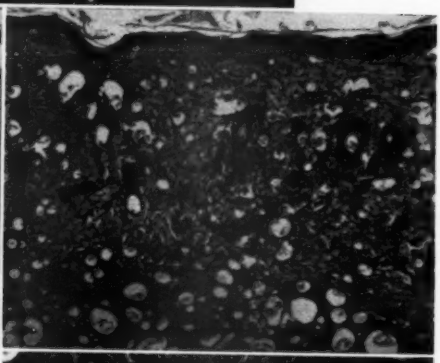
Fig. 7. A lymph node lesion shows multinucleated giant cells and focal suppuration around emulsion droplets (round spaces) that contained many acid-fast bacilli. Other areas exhibited epithelioid cell granulomas. × 125.



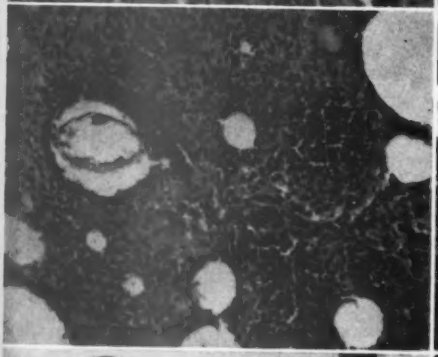


1

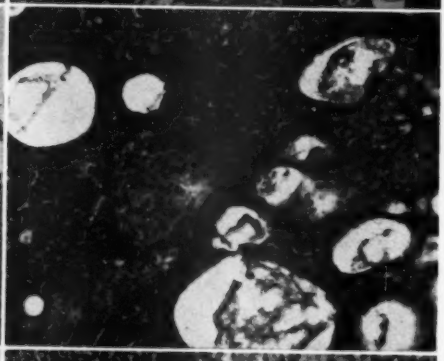
2



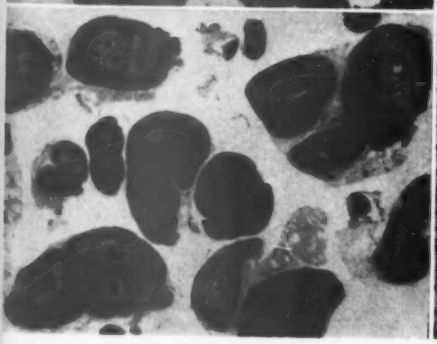
3



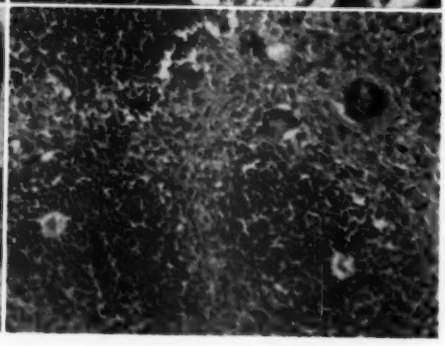
4



5



6



7

FIG. 8. Lung. An epithelioid cell granuloma with a multinucleated giant cell. Lung granulomas contained occasional acid-fast bacilli and were frequent when the adjuvant consisted of large amounts of tubercle bacilli, or after pertussis pretreatment, or in highly susceptible strains. × 500.

FIG. 9. An epithelioid cell granuloma in the spleen. Splenic granulomas increased in frequency as the level of tubercle bacilli in adjuvant was increased. Acid-fast bacilli were rare. × 500.

Figures 10 to 13 show lesions in the skin and draining lymph nodes following intradermal inoculation with encephalitogenic emulsion containing incomplete adjuvant (i.e., without tubercle bacilli).

FIG. 10. A rat from a strain with high susceptibility to EAE. Granulomatous inflammation appears at the inoculation site, even in the absence of tubercle bacilli. × 125.

FIG. 11. A rat from a strain with low susceptibility to EAE. There is a paucity of inflammation at the inoculation site. × 125.

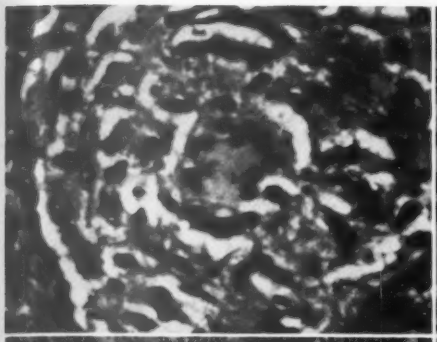
FIG. 12. A rat from a strain with high susceptibility to EAE. Granulomatous inflammation surrounds emulsion droplets in a draining lymph node. In this instance, tubercle bacilli are absent. × 125.

FIG. 13. Rat from a strain of low susceptibility to EAE. There is a paucity of inflammation around droplets of emulsion in a draining lymph node. × 125.

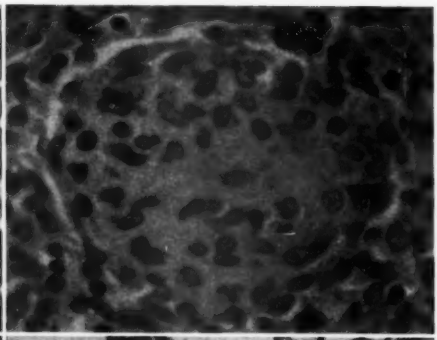
FIG. 14. A lung granuloma with a suppurative center and droplets of emulsion that contains many acid-fast bacilli. This lesion followed inoculation of an emulsion with a large dose of tubercle bacilli. × 125.

FIG. 15. A popliteal lymph node draining a foot pad inoculation site; the inoculum was an emulsion with complete adjuvant. There are massive enlargement, broad pale zones of confluent epithelioid granulomas, and innumerable small round spaces representing emulsion droplets. The foot pad route was highly efficient in producing EAE as well as granulomatous and suppurative inflammation in the regional lymph node. × 7.

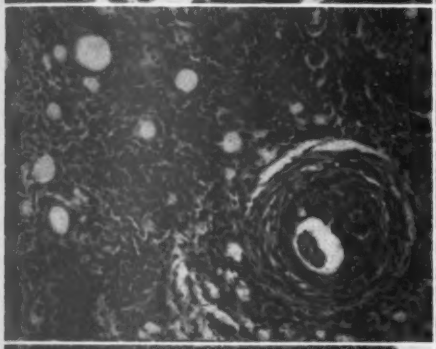
8
10
12
14



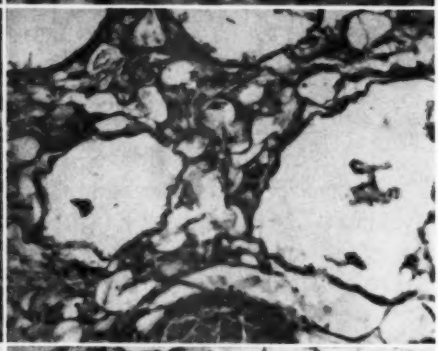
8



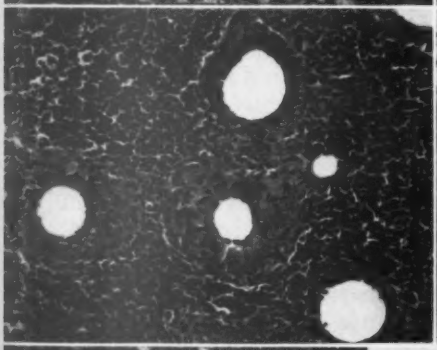
9



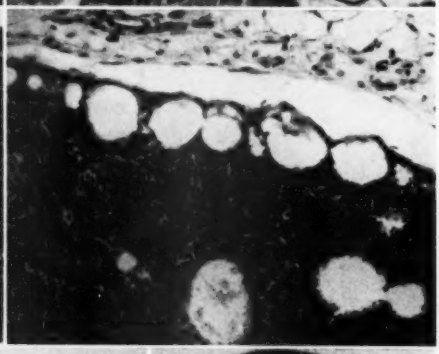
10



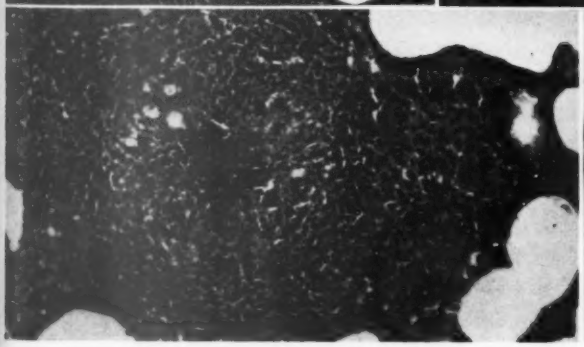
11



12



13



14

15

THE HISTOLOGIC DEMONSTRATION OF IRON IN OSSEOUS TISSUE

WALLACE L. PAST, M.D.

From the Department of Pathology, University of Louisville School of Medicine, Louisville, Ky.

There is little information available concerning the incorporation of iron into osseous tissue, though numerous other elements have been investigated for this characteristic. Von Gierke[1] in 1902 reported the discovery of iron in the bones of human and various animal embryos. Using an acid ferrocyanide solution, he obtained positive reactions consistently in ossification centers. He could not demonstrate iron in extrauterine specimens, and it was his impression that iron was substantially reduced in these if not completely absent. MacCallum[2] stated that if bones were "kept for a time in formalin," iron could be demonstrated along the line of ossification and beneath the periosteum. He denied that this iron represented impurities from the fixative. Quantitative chemical analyses by Austoni, Rabinovitch and Greenberg[3] revealed small amounts of nonhemoglobin iron in the osseous tissues of normal rats. These animals weighed 200 to 300 gm., and their tissues were freed from blood by viviperfusion. Marrow and osseous tissues were carefully separated before analysis.

The author's interest in the subject of iron in bone was stimulated by the following observation: A positive histochemical reaction for iron was found in the femoral trabeculae in a rat which had received a single injection of iron-dextran complex 3 days prior to sacrifice. This bone had been fixed in cold neutral buffered formalin and decalcified in formic acid-sodium citrate solution for about 3 days. It seemed possible that since iron is frequently present in pathologic calcifications,[4] it might in some circumstances also be involved in physiologic calcification. Preliminary investigation gave promise that the chelating agent, ethylenediamine tetra-acetic acid (EDTA), might, under appropriate conditions of pH, serve as a decalcifying agent which would leave tissue iron deposits *in situ*. The present investigation describes the development of such a decalcifying agent and the application of it to the experimental study of iron uptake by growing bones.

This work was supported in part by research grant RG-7329 from the National Institutes of Health, United States Public Health Service, Bethesda, Md.

Accepted for publication, May 4, 1961.

Material and Methods

Albino male rats of the Wistar strain were used. They were fed Ralston Purina Chow and weighed from 200 to 300 gm. at the time of injection. The injection substance was an iron-dextran complex (Imferon).

As decalcifying solutions, 5 per cent ethylenediamine tetra-acetic acid (EDTA) was prepared with pH's ranging from 7 to 12.5, adjusted with 1 N NaOH. A Beckman "G" pH meter was used.

Qualitative Tests on Decalcifying Solutions

Test for Ca^{++}. One ml. of 5 per cent sodium oxalate was added to 1 ml. of decalcifying solution. To the mixture, 1 N hydrochloric acid (HCl) was added, drop by drop, until maximum whitish turbidity developed. This was the positive reaction occurring in solutions of 3 mg. per cent or stronger.

Test for Fe^{+++}. Five drops of concentrated HCl were added to 1 ml. of decalcifying solution. One ml. of 2 per cent potassium ferrocyanide was then run slowly down the side of the test tube. The positive reaction was a blue "ring" developing at the interface, and this occurred in solutions of 0.06 mg. per cent or stronger.

Test for Fe^{++}. One ml. of 20 per cent potassium ferricyanide was added to 1 ml. of decalcifying solution. To the mixture, concentrated HCl was added, drop by drop, until maximum blue-green color developed. This was the positive reaction occurring in solutions of 0.2 mg. per cent or stronger.

The qualitative tests described above were worked out with positive controls, progressively diluted. Under the test conditions, the sensitivities were essentially the same whether the test material was in aqueous solution or complexed with EDTA.

Histochemical Tests

Test for Fe^{+++}. Histologic sections were immersed in a freshly prepared solution composed of equal parts of 2 per cent potassium ferrocyanide and 2 per cent HCl, at room temperature for 30 minutes. The counterstain was safranine.

Test for Fe^{++}. Histologic sections were immersed in a freshly prepared solution composed of equal parts of 20 per cent potassium ferricyanide and 1 per cent HCl at room temperature for 30 minutes. A safranine counterstain was used.

Experimental Procedure

Experiment I. This experiment was designed to find a decalcifying solution which would leave tissue iron deposits *in situ*. The animals were given a single intravenous dose of iron-dextran complex providing 500 mg. of Fe per kg. of body weight. They were sacrificed 3 days later by a blow to the head. Bones and soft tissues were fixed in cold neutral buffered formalin. Blocks of liver tissue as well as femurs were placed in EDTA solutions of different pH. Twenty-five ml. of solution were used per gm. of tissue. Every 2 days each solution was tested for calcium and iron and was then replaced with fresh solution. Bones were also blotted dry and weighed every 2 days on a torsion balance. Histologic sections were stained for iron.

Experiment II. The purpose of this experiment was to follow the localization of iron in the femurs of growing animals at various time intervals after injection. Iron was administered as above, and animals were sacrificed at intervals of 1 hour, 1 day, and 1, 2, 4, 8, 12, 16 and 55 weeks after injection. Another animal received 2 injections, 1 at 6 weeks and 1 at 3 weeks prior to sacrifice. The fixed femurs were decalcified in 5 per cent EDTA, pH 12, with testing and changing of each solution every 2 days as in experiment I. Again, histologic sections were stained for iron.

Results

Experiment I

Tables I and II illustrate the loss of iron from iron-laden liver tissue placed in 5 per cent EDTA solutions with pH's ranging from 7.1 to 12.5

and 10.8 to 12.3, respectively. The loss of iron diminished progressively as the alkalinity of the EDTA solution increased. At pH 12 the loss was nil, and the histochemical reaction for iron was maximal. In iron-laden femurs the findings were similar. Decalcification of the femurs required from one to several weeks. The decalcification time varied directly with the weight of the femur and was apparently unrelated to the pH of the

TABLE I

EXPERIMENT I. REMOVAL OF IRON FROM IRON-LADEN LIVER TISSUE BY EDTA DECALCIFYING SOLUTIONS (pH 7.1 TO 12.5). QUALITATIVE TESTS FOR Fe^{+++}

	Decalcifying solutions: 5% EDTA				
	Days				
pH	0–2	2–4	4–6	6–8	Histologic sections
7.1	3+	2+	1+	Neg.	2+
8.5	2+	Trace	Neg.	Neg.	2+
9.3	2+	Neg.	Neg.	Neg.	3+
10.0	2+	Neg.	Neg.		3+
11.8	1+	Neg.	Neg.		3+
12.5	Neg.	Neg.	Neg.	Neg.	4+

TABLE II

EXPERIMENT I. REMOVAL OF IRON FROM IRON-LADEN LIVER TISSUE BY EDTA DECALCIFYING SOLUTIONS (pH 10.8 TO 12.3). QUALITATIVE TESTS FOR Fe^{+++}

	Decalcifying solutions: 5% EDTA				
	Days				
pH	0–2	2–4	4–6	6–8	Histologic sections
10.8	2+	Neg.	Neg.		3+
11.4	2+	Neg.			3+
11.8	1+	Neg.			3+
12.0	Neg.	Neg.	Neg.		4+
12.2	Neg.	Neg.			4+
12.3	Neg.	Neg.			4+

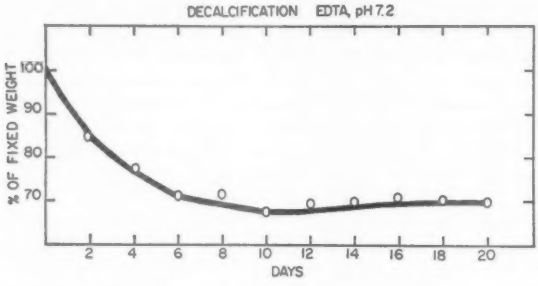
decalcifying solution. Text-figure 1 shows a typical decalcification curve in which the pH was 7.2. Text-figure 2 indicates that there was no iron loss from a femur decalcified at pH 12. Histochemical tests for iron revealed a strong positive reaction in the distal epiphysial plate and adjacent newly formed metaphysial trabeculae.

Experiment II

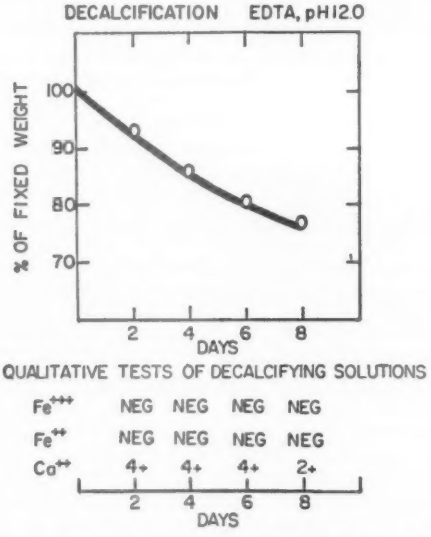
One hour after injection there was a strong positive reaction for iron in the distal epiphysial plate. This was localized to the hypertrophic cartilage cells (Figs. 1 and 2). There was also a weakly positive reaction in the osteoblasts and surrounding osseous tissue of the newly forming metaphysial trabeculae. One week after injection the trabecular reaction was stronger, but the cartilage cells gave a negative response (Figs.

3 and 4). At two weeks there was a negative zone between the epiphysial plate and the iron-positive metaphysial trabeculae, and some of the osteoblasts within the latter had become negative (Figs. 5 and 6).

A reaction in the osseous tissue of the diaphysial cortex was not encountered until 1 week following injection when it appeared immediately beneath the periosteum (Fig. 7). In succeeding weeks this "blue line" of iron positivity appeared to move progressively deeper into the cortex as iron-negative new bone formed on top of it (Fig. 8). Fifty-five weeks after injection the blue line was found slightly over half way



TEXT-FIGURE 1. Experiment I. Typical decalcification curve with 5 per cent EDTA. The original fixed weight of this rat femur was 0.65 gm.



TEXT-FIGURE 2. Experiment I. There is no detectable loss of iron from this femur decalcified in 5 per cent EDTA, pH 12. The original fixed weight of this rat femur was 0.72 gm.

through the cortex. This line was just as dark and thin as it had been at shorter intervals. The animal receiving two injections showed two parallel blue lines within the diaphysial cortex (Fig. 9).

Relatively little impairment of histologic sections or stains was observed even after prolonged soaking in the pH 12 solution. However, the metachromatic reaction of cartilage to the safranine counterstain was absent. One femur was decalcified prior to formalin fixation. Though the marrow was destroyed, a typical diaphysial blue line was present. Histologic sections of bone were consistently positive for Fe+++ but negative for Fe++.

Discussion

EDTA solution at pH 12, in contrast to conventional decalcifying agents seems to permit a selective removal of calcium from tissues. From a consideration of stability constants alone, one would expect EDTA to bind Fe+++ in preference to Ca++. However, at pH 12 the opposite situation obtains: EDTA will not remove iron from the highly insoluble ferric hydroxide, though its binding of calcium appears unaltered.[5]

The intravenous injection into rabbits of large doses of iron-dextran complex has been shown to give high serum iron levels which fall rather gradually to normal in about 10 days.[6] Therefore, it may be assumed that the growing bones had about 10 days' "exposure" to high iron levels immediately following injection.

In the present study it appeared that localization of iron in osseous tissue was highly accurate, since iron-positive bone was often sharply demarcated from iron-negative bone. The rounded foci of iron positivity noted in metaphysial trabeculae one week after injection, for example, were well defined. Furthermore, the central negativity exhibited by some of these foci at two weeks—which might have been predicted on the basis of a termination of the "exposure" period described above—provided additional evidence of accurate localization.

Since iron was incorporated at the epiphysis and also at the periosteal surface, it may be said that iron participated in both endochondral and membranous types of bone formation. In the former the so-called zone of provisional calcification became a zone of provisional "ferrification." Apparent movement of iron in the bones resulted from growth in length and diameter of these structures. This apparent movement resembled that previously described in radioautographic studies employing radioactive calcium and phosphorus.[7]

The experimental results raise the following questions: What is the exact location of iron in osseous tissue? What is its chemical form? Is iron incorporated in osseous tissue under physiologic conditions? In-

vestigations dealing with these problems are now under way in this laboratory. Also a variety of human pathologic calcifications are being investigated histochemically for iron localization.

Summary

Using 5 per cent ethylenediamine tetra-acetic acid (EDTA) at pH 12 it was possible to decalcify bones without removing tissue iron deposits. This permitted a histochemical study (Prussian blue reaction) of the uptake of iron by the femurs of young growing rats. The rats received single large intravenous doses of iron-dextran complex and were sacrificed at intervals ranging from 1 hour to 55 weeks thereafter. An apparent movement of iron resulted with bone growth during the intervals.

One hour after injection there was a strong reaction for iron in the hypertrophic cartilage cells of the distal epiphysial plate and a weak reaction in the newly forming trabeculae of the adjacent metaphysis. One week later the trabecular reaction was strong and sharply localized to osteoblasts and a narrow zone of surrounding osseous tissue. Thereafter, incorporation of iron into bone appeared to cease.

In the diaphysial cortex, iron first became evident one week after injection as a "blue line" immediately subjacent to the periosteum. In animals sacrificed at later intervals this line appeared to move progressively deeper into the cortex as the bones increased in diameter by accretional growth. Fifty-five weeks after injection the blue line was still present and its appearance was unchanged.

References

1. Gierke, E. Ueber den Eisengehalt verkalter Gewebe unter normalen und pathologischen Bedingungen. *Virchows Arch. path. Anat.*, 1902, **167**, 318–351.
2. MacCallum, W. G. A Textbook of Pathology. W. B. Saunders Co., Philadelphia and London, 1940, ed. 7, pp. 115–116.
3. Austoni, M. E.; Rabinovitch, A., and Greenberg, D. M. The iron content of the tissues of normal, anemic and iron-enriched rats freed from blood by viviperfusion. *J. Biol. Chem.*, 1940, **134**, 17–26.
4. Bunting, H. Histochemical analysis of pathological mineral deposits at various sites, with discussion of methods used. *Arch. Path.*, 1951, **52**, 458–469.
5. Sequestrene Technical Bulletin. Alrose Chemical Company, Providence, R.I., 1952, pp. 4–9.
6. Martin, L. E.; Bates, C. M.; Beresford, C. R.; Donaldson, J. D.; McDonald, F. F.; Dunlop, D.; Sheard, P.; London, E., and Twigg, G. D. The pharmacology of an iron-dextran intramuscular haematinic. *Brit. J. Pharmacol.*, 1955, **10**, 375–382.
7. Leblond, C. P., and Greulich, R. C. Autoradiographic Studies of Bone Formation and Growth. In: The Biochemistry and Physiology of Bone. Bourne, G. H. (ed.) Academic Press, Inc., New York, 1956, pp. 325–358.

The author is indebted to Mr. Floyd Poore and Mr. Charles Eversole for their interest

and suggestions in this investigation, and to Miss Hazel Short and Miss Joyce Rivers for their valuable technical assistance.

Iron-dextran complex (Imferon) was generously supplied by Lakeside Laboratories, Milwaukee, Wis.

[*Illustrations follow*]

Legends for Figures

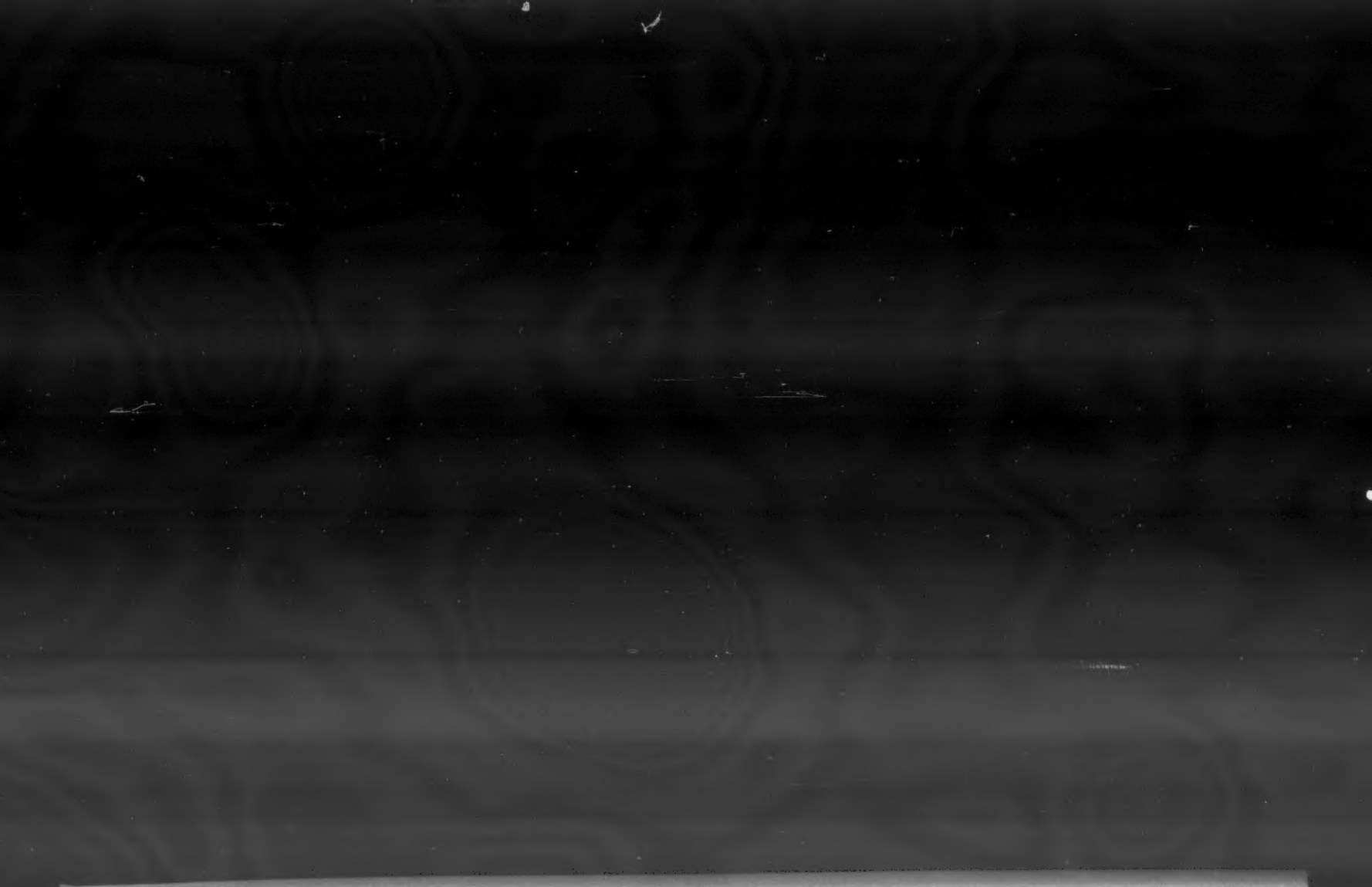
The photomicrographs illustrate the findings in experiment II in which rats were sacrificed at various time intervals after a single injection of iron-dextran complex. The femurs were decalcified in 5 per cent EDTA solution, pH 12, and histologic sections thereof were subjected to the Prussian blue reaction with safranine counterstain.

Fig. 1. At 1 hour after injection there is a strong positive reaction for iron in the hypertrophic cartilage cells of the distal epiphysial plate. A weak reaction appears in adjacent newly formed metaphysial trabeculae. × 100.

Fig. 2. A higher power view of an area shown in Figure 1. × 430.

Fig. 3. At 1 week the cartilage cells are negative for iron. The trabeculae have sharply defined rounded foci of iron positivity, and the marrow reacts strongly for storage iron. × 100.

Fig. 4. A higher power view of an area shown in Figure 3. × 430.



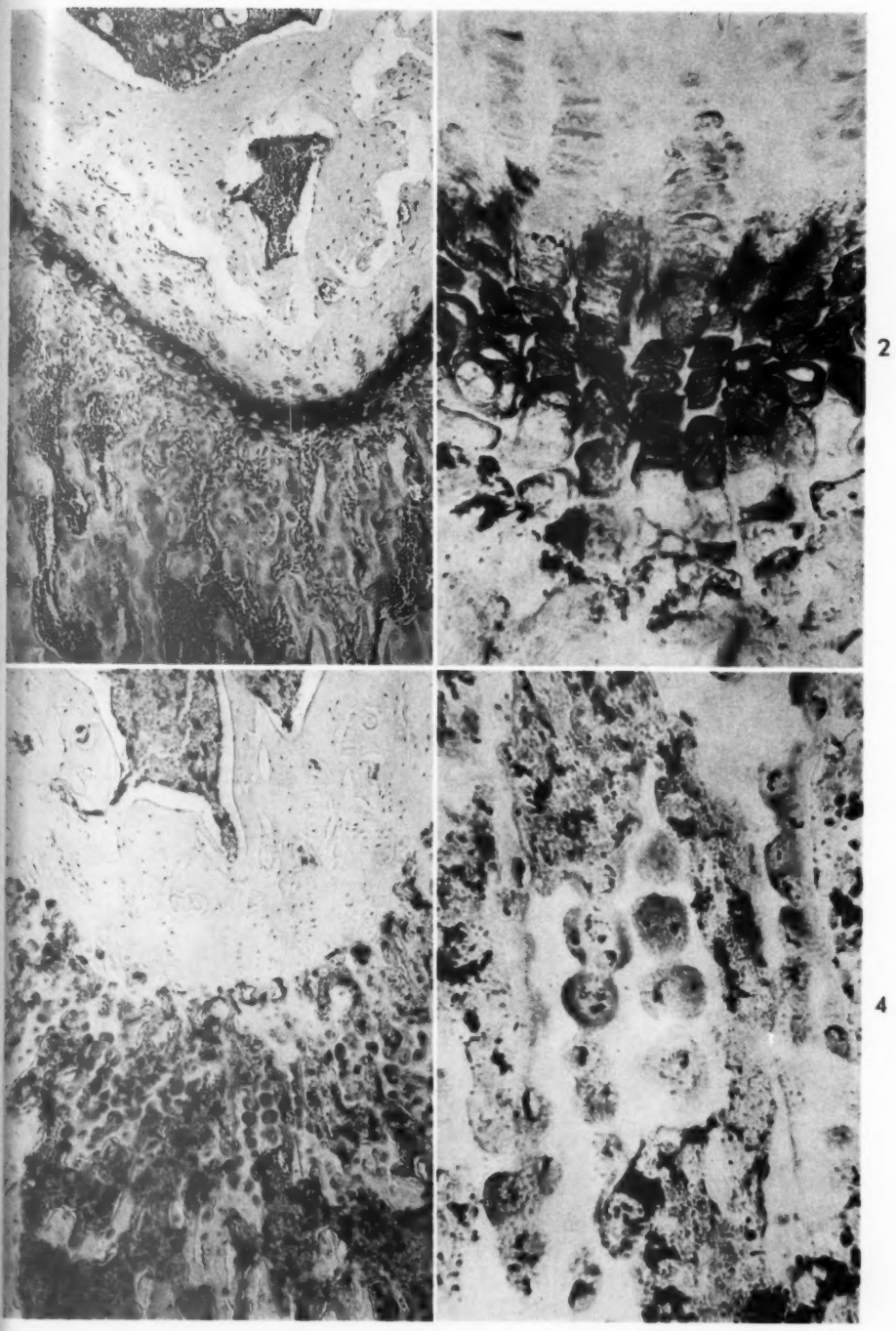


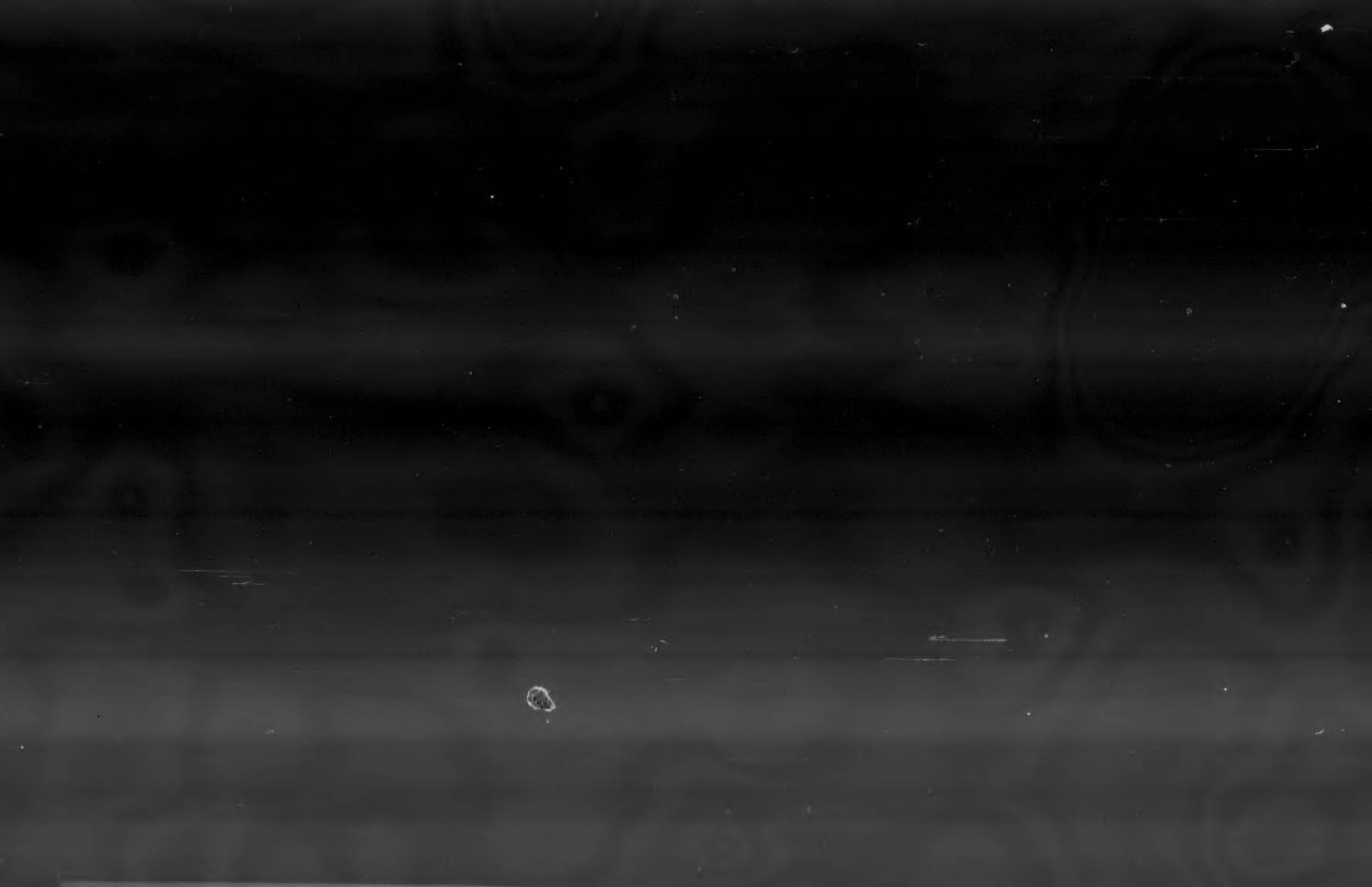
FIG. 5. At 2 weeks there is a zone of iron negativity adjacent to the epiphysial plate. × 100.

FIG. 6. Two weeks after injection there is a tendency toward "central clearing" of some of the rounded foci of positivity depicted in Figure 4. This results when iron-negative bone appears within such foci. The osteoblasts also tend to revert to negativity. × 430.

FIG. 7. At one week there is an iron-positive "line" beneath the periosteum of the cortical diaphysis. × 100.

FIG. 8. At 4 weeks the iron-positive "line" appears to have moved deeper into the cortex. × 430.

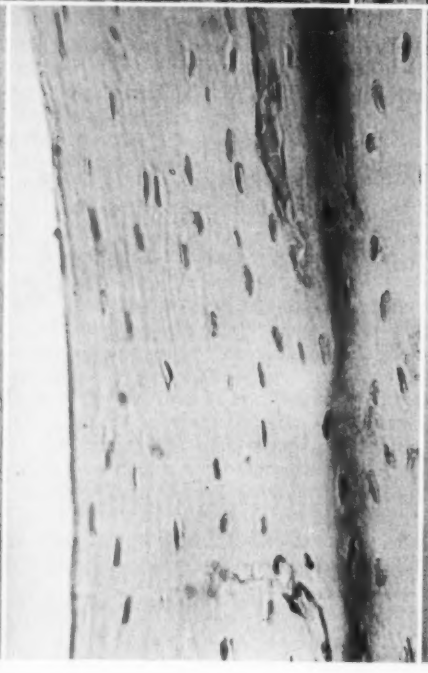
FIG. 9. Two lines are observed in the cortical bone of this animal which received 2 injections of iron, 1 at 6 weeks and 1 at 3 weeks prior to sacrifice. × 100.



5

6

7



8, 9

THE DELAYED EFFECTS OF EXTERNAL GAMMA IRRADIATION ON THE BONES OF RATS

RENATO BASERGA, M.D.; HERMANN LISCO, M.D., AND DONALD B. CATER, M.D.

From the Department of Pathology, Northwestern University Medical School, Chicago, Ill., the Argonne National Laboratory, Argonne, Ill., and the Department of Radiotherapeutics of the University of Cambridge, England

Ionizing radiations have been known for a long time to produce severe damage in bone, ranging from osteitis to necrosis, stunting of growth, pathologic fractures and malignant tumors.[1] The histologic features of irradiated bones were reviewed by Gates in 1943,[2] and since then there have been many reports concerning the effects of radiation on bone, especially from bone-seeking isotopes.[3] In a previous paper,[4] we reported the results of our investigations on the induction of tumors in rats in which both knee joints were irradiated with 3,000 r gamma rays from a source of iridium-192, and the effect of hormonal treatment upon the incidence of the radiation-induced tumors. The present paper deals with the pathologic changes that occurred in the irradiated bones and the relation of these alterations to the histogenesis of the neoplasms.

METHODS

A total of 170 female white Wistar rats, 60 days old and weighing from 100 to 130 gm., were used. Both knee joints of 136 rats were irradiated with a single dose of 3,000 r gamma radiation from an iridium-192 source; the details of this procedure have been described in an earlier paper.[4] The field of irradiation included the epiphyses and a portion of the bone shaft of the femur, tibia and fibula. Thirty-four rats from the same litters were allocated to the unirradiated control group. Both control and irradiated animals were randomized into 4 groups, which received, respectively, injections of saline, growth hormone, thyroxin, and growth hormone and thyroxin, as described previously.[4]

All rats were weighed once a week, and diagnostic radiographs were made at 185, 280 and 386 days and before necropsy. The earliest death occurred 49 days after irradiation, and the last experimental animals were sacrificed 755 days after the beginning of the experiment. There was some loss of experimental animals in the course of the investigation, but in the end, tissue from 112 irradiated and 28 nonirradiated rats was available for histologic study. The mean age, at death, was 525 days.

The lengths of the femurs and tibias were measured with a standard ruler on the roentgenograms which were all taken at the same focal distance. In only a few instances the position of the leg was such as to make the measurement unreliable, and such cases were discarded from the computations. At necropsy, samples were taken routinely from most organs and from any grossly visible lesions. Tissue was fixed in SUSA, and sections were stained with hematoxylin and eosin. Both legs and one forelimb of each rat were fixed in formalin; after dissection they were decalcified with nitric acid (3 per cent), embedded in celloidin, sectioned and stained with

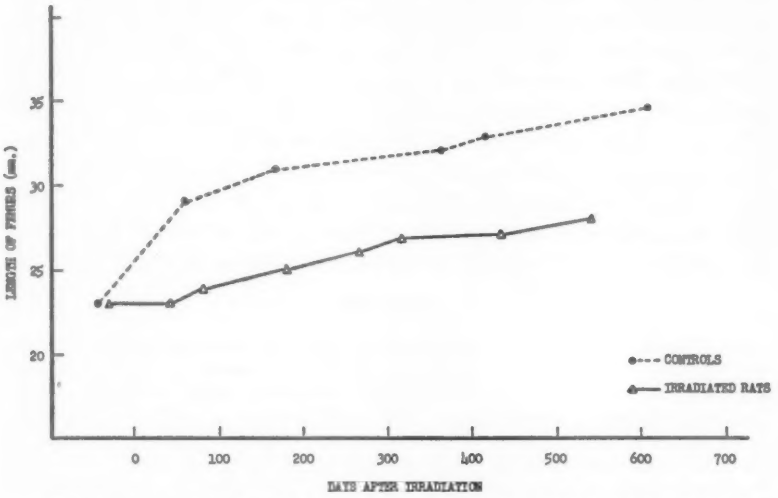
Accepted for publication, May 4, 1961.

hematoxylin and eosin. Special stains were used when indicated. All sections were studied by repeated blind readings until consistent descriptions and interpretations were obtained. An eyepiece micrometer was used to measure the thickness of the epiphysial cartilage plates. As the thickness of a given cartilage plate varied considerably from one point to another, it was arbitrarily decided to take the maximum width toward the center as the standard measure of thickness. Micrometric measurements of the thickness of the articular cartilage and of bone growth were also made.

RESULTS

Analysis of Roentgen Films

The growth rate of the bones in irradiated and control rats, as measured by the length of the femurs in diagnostic radiographs is shown in Text-figure 1. It can be seen that the control rats showed an initial



TEXT-FIGURE 1. Growth rate in the femurs of control and irradiated female rats, 60 days old at the time of irradiation.

period of rapid growth. This was followed, toward the 200th day of the experiment, by a period of reduced growth rate, although a slow, steady growth persisted until the end of the experiment. At this time the mean length of the femurs was 33.4 mm. In the irradiated animals, bone growth, arrested immedately after irradiation, as observed by Hinkel[5] and by Barr, Lingley and Gall,[6] resumed after approximately 60 days.[7] However, the femurs in the irradiated rats remained shorter, and at the end of the experiment their mean length was 26.2 mm. It will also be noted that after the immediate period of complete or almost complete arrest, the growth rate of the irradiated femurs was similar to that of the controls.

If one applies the calculations of Kember[8] to our material, a shortening of 7 mm. indicates that the cartilage cells of the epiphysial plates have missed, during the 60 days in which growth was arrested, a total of approximately 280 mitotic cycles. After this, cell division, and therefore growth, was resumed. The phenomenon of delayed mitotic recovery is well known, and it has been recently reviewed by Scanlon.[9]

Similar changes were observed in the growth rate of the tibias; at the beginning of the experiment the mean length of these was 28.5 mm., and at the end it was 29.6 mm. in the irradiated animals and 36 mm. in the control group. The results are summarized in Table I; only those

TABLE I

MEAN LENGTH AND STANDARD DEVIATIONS OF THE FEMURS AND TIBIAS OF RATS MORE THAN 300 DAYS OLD

	Right femur	Right tibia	Left femur	Left tibia
Irradiated rats	26.1 ± 3.0	29.8 ± 3.4	26.3 ± 2.0	29.5 ± 3.1
Nonirradiated rats	33.4 ± 1.0	36.2 ± 1.0	33.4 ± 1.7	35.8 ± 1.4

animals that died or were sacrificed after 300 days from the beginning of the experiment are included in the table. The differences between the control and the irradiated rats are significant ($p<0.001$).

Among the irradiated rats, the differences between the femurs or the tibias of the two sides are not significant, but the differences in shortening between the femurs and the tibias must be accepted as significant (for the right limb, $t=2$, $n=203$, $p<0.05>0.025$; for the left, $t=2.2$, $n=201$, $p<0.05>0.025$). It should be noted that in growing rats the proximal cartilage plate contributes more than 75 per cent of the total growth of the tibia,[8] so that the residual growth of the irradiated tibia can be ascribed to the activity of the nonirradiated distal plate. This explanation cannot be applied to the femurs, as will be shown presently. Qualitative radiologic changes in the irradiated bones have been described previously.[10]

Histologic Features in Control Bones

The bones of nonirradiated animals showed changes consistent with those of increasing age.[11] The cartilage cells were regular and grouped in orderly conical masses (Fig. 1), with a moderate amount of intercellular matrix. In the older animals, both the femoral and tibial epiphysial plates were sealed by a thick lamina of bone. In the younger ones, the lamina was incomplete, and the epiphysial plates were separated from the marrow cavity by a rim of fibrous tissue, or continued directly into the metaphysial trabeculae.

The mean thickness of the epiphysial cartilage plates in the femurs

and tibias of both legs is shown in Table II. An analysis of the thickness of the cartilage plates as a function of time showed that the values,

TABLE II

MEAN THICKNESS AND STANDARD DEVIATIONS OF THE EPIPHYSIAL CARTILAGE PLATES OF FEMURS AND TIBIAS OF RATS MORE THAN 300 DAYS OLD

	Right femur	Right tibia	Left femur	Left tibia
Irradiated rats	393 ± 111	313 ± 75	378 ± 122	320 ± 111
Nonirradiated rats	145 ± 38	140 ± 33	154 ± 28	146 ± 33

after the 300th day, were not significantly different. The number of animals that died before the 300th day was too small to allow any statistical treatment, and these animals were simply discarded from the computations. It will be noted that the mean value of the left femur is highest, and of the right tibia, lowest. The differences, however, are not statistically significant.

The metaphysial trabeculae decreased in number and size with age; islands of cartilage were occasionally found in them and in the cortical bone of the diaphysis. The bone marrow was mostly cellular and contained a small amount of fat. The number of nonirradiated rats treated with hormones was small, but the thickness of the cartilage plates was of the same order as that in the saline-treated animals, and the bones in the 4 groups could not be separated after repeated blind readings.

Histologic Features in Irradiated Bones

Epiphysial Cartilage Plates. Severe alteration of the epiphysial cartilage was a consistent feature in almost all of the irradiated animals.[2,6] Manifest were a loss of the orderly columnar orientation of the cartilage cells (Fig. 2), empty lacunas, bizarre cartilage cells, sometimes with eosinophilic cytoplasmic inclusions,[12] and marked variation in the size and shape of cells. The mean thickness of the cartilage plates of the femurs and tibias in both legs is shown in Table II, where the animals that died before the 300th day were, as indicated, discarded from the computations. The differences between the control and the irradiated rats were significant ($p < 0.001$). Among the irradiated rats, the differences between the femurs and the tibias of both sides were not significant, but the differences between the femurs and the tibias were (for the right limb, $t = 5.5$, $n = 172$, $p < 0.001$; for the left, $t = 3.2$, $n = 173$, $p < 0.005$).

Premature closure of the epiphysial plates, as revealed by the appearance of a bony lamina on the diaphysial side, a frequent finding in irradiated bones,[7] was found as early as 215 days after irradiation. Doubling of the cartilage plates, which was not observed in nonirradiated

animals, was found in 40 instances; it appeared as early as 274 days after irradiation, and the incidence increased with time. Double plates were either completely separated by marrow tissue or connected by cartilaginous trabeculae or spicules of bone (Fig. 3). In other instances, thickened plates were seen containing small islands of marrow surrounded by rims of bone; all intermediate stages were seen, from small islands of marrow, coalescing marrow spaces partially separating the plates, and frankly split cartilage plates. This seems to indicate that the doubling of the cartilage plates was due to secondary invasion of the thickened cartilage by blood vessels and bone marrow, a feature similar to the normal mechanism of capillary erosion and tunneling of cartilage in the formation of secondary ossification centers.[13] Once the plate was split by the coalescing marrow spaces, the growing part—that toward the epiphysis, containing the various layers of dividing cartilage cells[8]—might continue the process of longitudinal growth, thus pulling away from the other half not containing proliferating cartilage cells.

Spongiosa. The terms "old" and "new" spongiosa are used here in the sense given by Heller,[7] the former referring to the devitalized spongiosa affected at the time of irradiation. The new spongiosa developed between the old and the epiphysial cartilage; it constituted the morphologic evidence of the resumption of growth. A typical severance of the primary spongiosa[7,12,14,15] was found in two early specimens, 49 and 79 days after irradiation. As growth resumed, the severed spongiosa was left behind, and its fate could be followed roentgenographically and by examination of specimens at different intervals. In bones 185 days or more after irradiation, the devitalized spongiosa appeared in radiographs as a bar of increased density across the marrow cavity,[10] and in sections as a bony bar with a mixture of dead, fibrous and atypical bone (Fig. 4). Partial resorption of the devitalized bone produced a somewhat bizarre pattern (Fig. 5).

A combined study of roentgen films and sections showed that bony bars were present in 70 instances, all except one in the femur. In the older specimens, the distance of the devitalized spongiosa from the epiphysial plate averaged 3 mm. Applying the calculations of Kember,[8] a distance of 3 mm. represents about 120 cell divisions of the cartilage plate cells from the time of irradiation. In 42 instances a new spongiosa, with irregular, closely packed trabeculae of fibrous bone (Fig. 4), appeared between the epiphysial plate and the old spongiosa. The incidence of this newly formed, almost solid, spongiosa, probably the expression of an impaired resorption of bone in the irradiated tissues,[16,17] reached a peak between the 400th and 500th day after irradiation (50 per cent), then decreased first slowly (39 per cent between the 500th and 600th

days) and then rapidly (less than 10 per cent after the 600th day).

Cortical Bone. Changes in the cortical bone were surprisingly slight, and were limited to a few dead osteocytes at the level of the old devitalized spongiosa and to secondary alterations incident to the presence of tumors or pathologic fractures. Frequently, masses of cartilage were observed in the cortex of the shaft, but they were also found often in control rats.[11,18]

Articular Cartilage and Epiphysis. In comparison with the epiphysial cartilage, the articular cartilage suffered very little damage. In fact, the only feature worthy of note was encountered in a few rats that died early during the experiment. These showed some cellular activity in the articular cartilage, as revealed by closely packed cells in irregular layers and with scanty matrix. In older animals, fraying and focal degeneration of the articular cartilage were observed, but these were also found in nonirradiated rats.

The epiphysis showed a series of interesting lesions, the most conspicuous being fibrosis of the marrow spaces, formation of fibrous bone, and foci of osteosclerosis. These were in addition to bone tumors to be described later. Such lesions were also seen in the metaphysis and, occasionally, in the diaphysis of the irradiated bones, but they were considerably more frequent and more pronounced in the epiphysis.

Bone Marrow. In all 10 rats that died before the 150th day of the experiment, the bone marrow in both hind extremities was gelatinous. Cells of both the erythrocytic and granulocytic series reappeared in the marrow after the 273rd day, but extensive regions of gelatinous or fibrous marrow (Fig. 6) were still present, even in those animals sacrificed after the 700th day. The incidence, however, decreased sharply with time.

Other Bone Changes. Pathologic fractures, a not infrequent complication of external radiation,[19–21] were found in 15 rats. They were always observed in the femur, usually at the level of the old devitalized spongiosa. Exostoses were found in two instances. In 6 irradiated rats, cysts were encountered in the marrow cavity of the femur or tibia. These had a thin rim of loose fibrous tissue and contained an amorphous basophilic substance (Fig. 7) with occasional mononuclear cells. In most of the irradiated bones there was a marked decrease in vascularization. This was especially true when the concomitant injury was more severe, as in badly damaged epiphyses or in unresorbed spongiosas. In one instance, the metaphysial region of the femur contained numerous blood-filled dilated vessels resembling hemangiectases.[12]

Hormone treatment had no appreciable influence on the stunting of growth incident to irradiation, on the thickness of the cartilage plates, or on other radiation-induced lesions.

Abnormal Bone. Fibrous or coarse-fibered, or woven bone is found in the embryo and appears in all sorts of pathologic conditions where new bone is rapidly formed.[22] It corresponds to the atypical bone of Looney[23] and the fibrillar connective tissue heavily impregnated with calcium salts of Jaffe.[24] It appears as a coarsely fibrous, blue-staining bone, sometimes surrounded by lamellar bone, and is basophilic in decalcified preparations.[16] The basophilic properties are due to the abundance of cement, which contains mucopolysaccharides.[18] In our specimens, coarsely fibrous bone was present (Fig. 8) in almost 100 per cent of the cases, more often in the femur and tibia, but frequently also in the patella (Fig. 9). Its incidence reached a peak between the 400th and 600th days, and decreased slowly thereafter.

We have taken "atypical bone" to signify the presence of osteoid deposits in the marrow spaces, and of spicules of bone forming irregular masses with little tendency to construct orderly trabeculae (Fig. 10). Atypical bone was usually found in connection with dead or fibrous bone, and in the epiphysis it had no relation to pre-existing trabeculae.

Areas of bone sclerosis were characterized by masses of well-formed and well-differentiated bone trabeculae,[10] closely packed together with narrow marrow spaces. Large amounts of dead bone were found at the level of the old spongiosa and, to a less extent, in the epiphysis.

Histologic Characteristics of Tumors

Aside from epidermoid carcinoma of the skin and glandular carcinoma of the mammary gland, the tumors found in the field of irradiation could be divided into two categories: bone-forming tumors, i.e., osteosarcomas, and a variety of sarcomas not producing bone, which, for the sake of simplicity, we shall call fibrosarcomas. As far as the origin of these tumors is concerned, it may be safely stated that all osteosarcomas arose within the bone. Fibrosarcomas, however, could be divided into 3 subgroups: (a) those originating from the soft tissue of the leg, in which the tumor cells came into no contact with the bone; (b) those in which the tumor cells came into contact with the bone but did not invade it. Some of these were obviously of periosteal origin, but others were too huge to permit an evaluation of their origin; and (c) those that had replaced large portions of bone. In these an origin from within the bone could not be ruled out. Arbitrarily, it was decided to consider as bone tumors only the osteosarcomas. It should be emphasized, however, that simple considerations of the mechanics of invasion[24] strongly suggest that the fibrosarcomas in group 3 actually arose within the bone.

Osteosarcoma. A total of 34 osteosarcomas were observed. As reported previously,[4] the incidence was higher in rats treated with growth hormone, and the induction period was shorter in those treated with

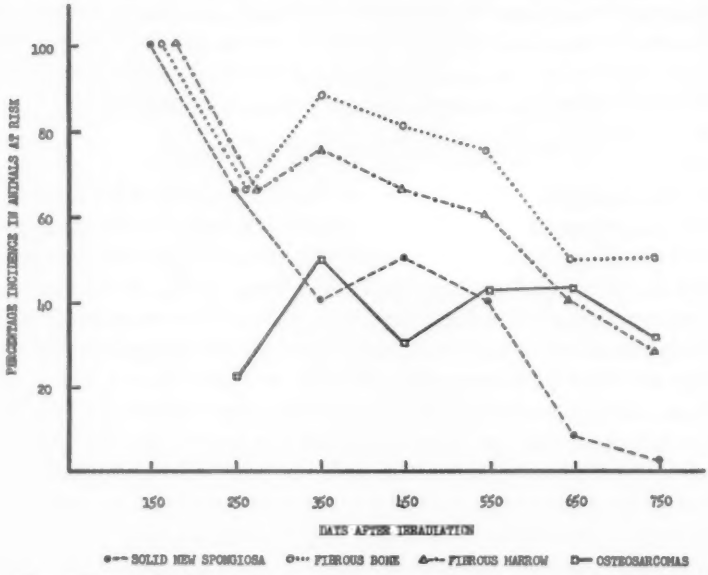
thyroxin (Table III). Their chronologic and topographic distribution are shown in Text-figure 2 and Table IV respectively. The neoplasms

TABLE III

INCIDENCE OF OSTEOSARCOMA AND FIBROSARCOMA IN RATS AFTER LOCAL IRRADIATION AND HORMONE TREATMENT

	Osteosarcoma			Fibrosarcoma		
	Incidence	Per-centage	Latent period	Incidence	Per-centage	Latent period
Gamma rays and growth hormone	12/30	40	520 ± 49	12/30	40	465 ± 36
Gamma rays and thyroxin	8/29	27.5	388 ± 29	9/29	31	468 ± 33
Gamma rays, growth hormone and thyroxin	7/26	27	512 ± 35	12/26	46	445 ± 50
Gamma rays and saline	7/31	22.5	521 ± 46	17/31	4.7	441 ± 25
Totals	34/116	29.3	488 ± 44	50/116	43.1	453 ± 36

ranged in size from microscopic lesions to large masses measuring 40 mm. in maximum dimension. In 7 instances they were found to arise



TEXT-FIGURE 2. Incidence of osteosarcoma and non-neoplastic lesions in locally irradiated rat bone, at various intervals after a single dose of 3,000 r of gamma rays.

from the epiphysis, and in 5 instances from the metaphysis. In 22 cases the tumors were of such size when first detected by roentgenograms or on histologic examination that the determination of the exact site of origin was impossible.

Invasion of epiphysial or articular cartilage or erosion of bone cortex was *conditio sine qua non* to the diagnosis of osteosarcoma. Accordingly, smaller nests of atypical bone formation were not grouped among the tumors although it is likely that at least some of them may have represented early neoplastic transformation. Small nests of this nature were found more frequently in the metaphysis (Fig. 11), and micrometric measurements showed that they were located about 3 mm. from the bony bars of old devitalized spongiosa. No tumors were found to arise from the patella. This bone, however, frequently showed signs of radiation damage (Fig. 9).

Fibrosarcoma. A total of 50 fibrosarcomas were induced in the 116 rats receiving gamma rays. This incidence is comparable with that reported by Koletsky and Gustafson,[25] using 660 r of whole-body x-irradiation, and supports the opinion that postirradiation sarcomas of soft tissues are not a rarity.[26–28] The tumors ranged in size from microscopic lesions to large masses measuring 55 mm. in maximum dimension. For the most part, they were well differentiated, but 7 were undifferentiated, anaplastic and markedly pleomorphic. The topographic distribution is shown in Table IV.

TABLE IV

DISTRIBUTION OF OSTEOSARCOMA, FIBROSARCOMA AND NON-NEOPLASTIC LESIONS IN THE FEMURS AND TIBIAS OF IRRADIATED RATS

	Right femur	Right tibia	Left femur	Left tibia
Osteosarcoma	10	3	15	6
Fibrosarcoma	23	2	25	4
Solid spongiosa	19	3	8	7
Bone sclerosis	25	16	20	12
Split cartilage plate	5	11	8	16
Epiphysial cartilage thickness (in μ)	393	313	378	320
Stunting of growth (in mm.)	7.3	6.4	7.1	6.3

Other Neoplasms. The following were also found in the irradiated rats[4]: 23 fibroadenomas and 12 glandular carcinomas of mammary gland origin, 10 skin carcinomas, 2 carcinomas of the thyroid, 1 lymphosarcoma of the mediastinum; in 2 instances there was myeloid leukemia.

DISCUSSION

Bone changes after external radiation have been described by many authors.[2,3,6,12,29–32] Although most previous investigations have been concerned with short postirradiation intervals, the present observations, in general, confirm earlier ones. The most striking changes in our animals

consisted in stunting of growth, marked alterations of cartilage cells in epiphysial plates, formation of fibrous and atypical bone, and lack of resorption of the newly laid spongiosa. Doubling of the epiphysial cartilage plates has not, to our knowledge, been previously reported, although Heller[7] described "severed" hypertrophic plates in rats 5 months after the injection of 0.5 μc. of radium.

Stunting of growth has been produced with as little as 665 r of x-rays,[6] but the degree of stunting appeared to depend not only on the dose but also on the age of the animal treated.[5] Micrometric measurements in the bones of our rats, taking the distance between the epiphysial plate and the old devitalized spongiosa as the morphologic indicator of growth, showed elongation of 3 mm. after irradiation. This constitutes a good growth but is still about 7 mm. less than that observed in nonirradiated rats. When expressed in terms of cellular division,[8,32] it may be taken to indicate that the irradiated cartilage cells in the epiphysial plates missed or were delayed for a time period equal to about 280 mitoses but were then still capable of undergoing about 120 cell divisions.

An obvious fact should be emphasized at this point; the metaphyses in irradiated animals, 3 or more months after radiation, contained many cells which had never been hit by the radiation itself. These were, in other words, the daughter cells of those which had been radiated. Considering the turnover rate of cells in the metaphysial bone of rats, as shown by Kember,[8] it is obvious that in studying the late effects of radiation, one is actually investigating the effects in the daughter cells, many generations removed from the cells actually irradiated. In this respect, bone, with its longitudinal growth, offers an interesting model in which the number of cell divisions from the time of irradiation may actually be counted. This is especially true of the cells in the cartilage plates. It is therefore a reasonable assumption that, whatever disturbances have been produced by radiation in bone or cartilage cells, these derangements have been handed down from cell to cell for several generations. Moreover, it would seem that the clones of modified cells have a tendency to become extinct, as the incidence of the changes produced by radiation decreases with time. *Restitutio ad integrum* in irradiated tissues would then depend on selective processes tending to eliminate modified cells, and the rate of such selection would express itself in the recovery rate from radiation.[33] This may have some bearing on the histogenesis of osteosarcomas. First, however, we would like to approach the problem of bone tumor induction by comparing the distribution, in time and space, of osteosarcomas to the distribution of nontumorous lesions in the bones of irradiated animals.

An analysis of Text-figure 2, which indicates the temporal distribu-

tion of osteosarcomas and some non-neoplastic lesions, reveals a rise in the incidence of osteosarcomas when the incidence of non-neoplastic lesions decreases. It should be noted that the inception of some of the osteosarcomas was determined by roentgenogram or clinical examination, while the appearance of non-neoplastic lesions was detected only by necropsy examination. Thus, the shift to the right shown by osteosarcomas is in reality more accentuated than appears in Text-figure 2. Again, it should be emphasized that the nontumorous lesions are expressions of a repair process following initial damage. Thus, an analysis of Text-figure 2 indicates that the incidence of osteosarcomas reaches a peak at a moment when restoration of the damaged bone is also a prominent feature.

The distribution of osteosarcomas and reparative lesions in the femurs and tibias is shown in Table IV. Although there is a good correlation between the distribution of osteosarcoma and fibrosarcoma, there is no correlation in the distribution of tumors and non-neoplastic lesions. This suggests that the mechanism triggering the initiation of both neoplasms is the same and is unrelated to the amount of damage.

Further attempts were made to correlate the incidence of tumors with the degree of radiation damage, as measured by the stunting of growth and the thickness of the epiphysial cartilage. The incidence of osteosarcoma is definitely higher in the femur, where the increase in thickness of the epiphysial plate is significantly higher than in the tibia. The difference in the stunting of growth is at the border line of statistical significance. This seems to lend some support to the idea of a correlation between tumor induction and the degree of radiation damage. However, when the tumor-bearing bones were compared with all the irradiated bones, the results were disappointing. The mean lengths of the tumor-bearing femurs was 25.6 ± 2.3 mm., and the tibias was 28.2 ± 2 mm. These were less than the values obtained from all irradiated rats, but not significantly so.

The situation is similar when the mean thickness of the epiphysial cartilage plates is taken as a measure of radiation damage. In femurs with tumors the mean thickness of the epiphysial plates was $314 \pm 70\ \mu$ (a value which is actually lower than that in all irradiated animals). In the tibias it is $320 \pm 85\ \mu$, the same as that of all irradiated animals. The degree of thickening of the epiphysial plates thus cannot be correlated with the incidence of neoplasms.

It should be noted that the differences in radiation damage or in the incidence of tumors in the femur and tibia are correlated to the growth rate of the two bones. In control animals, the femur grew 10.5 mm. from the beginning of the experiment; the tibia grew 7.5 mm. This seems

to indicate that the rate of bone growth may have some influence on the incidence of tumors. Further support to this hypothesis was gathered by the fact that no tumors were found to arise from the patella where, despite signs of severe radiation damage, the rate of cellular turnover is ordinarily modest.

In summarizing our observations, it appears that the time of occurrence and the location of osteosarcomas do not parallel the occurrence of non-neoplastic lesions and do not correlate with the degree of radiation damage, as measured by the stunting of growth and the thickening of the epiphysial cartilage plates. On the other hand, the distribution of tumors correlates with the growth rate of the affected bone, the incidence being higher in more rapidly growing bones.

Summary

The delayed effects of external radiation have been investigated in rats with both knee joints irradiated by 3,000 r of gamma rays from an iridium-192 source.

An attempt was made to correlate radiation damage and the incidence of bone neoplasm in the radiated area. The results indicated that under the conditions of the experiment, there was no correlation between the degree or the distribution of radiation damage and the incidence of tumors. A positive correlation was found to exist between incidence of tumors and the growth rate of tissue at the time of irradiation.

References

1. Brooks, B., and Hillstrom, H. T. Effect of roentgen rays on bone growth and bone regeneration; experimental study. *Am. J. Surg.*, 1933, **20**, 599–614.
2. Gates, O. Effects of radiation on normal tissues. XII. Effects on bone, cartilage and teeth. *Arch. Path.*, 1943, **35**, 323–340.
3. Vaughan, J. Radiation Injury to Bone. In: Lectures on the Scientific Bases of Medicine, 1954–1955. University of London, Athlone Press, London, 1956, Vol. 4, pp. 196–223.
4. Cater, D. B.; Baserga, R., and Lisco, H. Studies on the induction of bone and soft tissue tumours in rats by gamma irradiation and the effect of growth hormone and thyroxine. *Brit. J. Cancer*, 1959, **13**, 214–227.
5. Hinkel, C. L. The effect of roentgen rays upon the growing long bones of albino rats; quantitative studies of growth limitation following irradiation. *Am. J. Roentgenol.*, 1942, **47**, 439–457.
6. Barr, J. S.; Lingley, J. R., and Gall, E. A. The effect of roentgen irradiation on epiphyseal growth; experimental studies upon the albino rat. *Am. J. Roentgenol.*, 1943, **49**, 104–115.
7. Heller, M. Bone. In: Histopathology of Irradiation from External and Internal Sources. Bloom, W. (ed.). McGraw-Hill Book Co., New York, 1948, Chapt. 5, pp. 70–161.
8. Kember, N. F. Cell division in endochondral ossification; a study of cell proliferation in rat bones by the method of tritiated thymidine autoradiography. *J. Bone & Joint Surg.*, 1960, **42 B**, 824–839.

9. SCANLON, P. W. The effect of mitotic suppression and recovery after irradiation on time-dose relationships and the application of this effect to clinical radiation therapy. *Am. J. Roentgenol.*, 1959, **81**, 433–455.

10. CATER, D. B.; BASERGA, R., and LISCO, H. Radiographic appearances of bone lesions in rats exposed to local external irradiation from gamma rays. *Acta radiol.*, 1960, **54**, 273–288.

11. BECKS, H.; SIMPSON, M. E., and EVANS, H. E. Ossification at the proximal tibial epiphysis in the rat. I. Changes in females with increasing age. *Anat. Rec.*, 1945, **92**, 109–119.

12. HINKEL, C. L. The effect of roentgen rays upon the growing long bones of albino rats. II. Histopathological changes involving endochondral growth centers. *Am. J. Roentgenol.*, 1943, **49**, 321–348.

13. HAM, A. W. Histology. J. B. Lippincott, Philadelphia, 1957, ed. 3, 894 pp.

14. CALDWELL, E. W., and BENNETT, G. A. Comparative histologic effects of 400 kilovolt and 23 million electron volt x-rays on the long bones of young albino rats. (Abstract) *Am. J. Path.*, 1952, **28**, 547.

15. POBEDINSKY, M. N. Development of Tumours in Animals under Influence of Penetrating Radiation. In: Progress in Radiobiology. Oliver & Boyd, London, 1956, pp. 433–435.

16. BLOOM, M. A., and BLOOM, W. Late effects of radium and plutonium on bone. *Arch. Path.*, 1949, **47**, 494–511.

17. OWEN, M.; SISSONS, H. A., and VAUGHAN, J. The effect of a single injection of high dose of ^{90}Sr (500–1000 μc./kg.) in rabbits. *Brit. J. Cancer*, 1957, **11**, 229–248.

18. PRITCHARD, J. J. General Anatomy and Histology of Bone. In: The Biochemistry and Physiology of Bone. BOURNE, G. H. (ed.). Academic Press, Inc., New York, 1956, pp. 1–25.

19. BAENSCH, W. Knochenschädigung nach Röntgenbestrahlung. *Fortschr. Geb. Röntgenstrahlen*, 1927, **36**, 1245–1247.

20. KOK, G. Spontaneous fractures of the femoral neck after the intensive irradiation of carcinoma of the uterus. *Acta radiol.*, 1953, **40**, 511–527.

21. STEPHENSON, W. H., and COHEN, B. Post-irradiation fractures of the neck of the femur. *J. Bone & Joint Surg.*, 1956, **38 B**, 830–845.

22. BAKER, S. L. Fibrogenesis imperfecta ossium; generalized disease of bone characterised by defective formation of collagen fibres of bone matrix. *J. Bone & Joint Surg.*, 1956, **38 B**, 378–417.

23. LOONEY, W. B. Late effects (25 to 40 years) of the early medical and industrial use of radio-active materials. 1. Their relation to more accurate establishment of maximum permissible amounts of radio-active elements in the body. *J. Bone & Joint Surg.*, 1955, **37 A**, 1169–1187.

24. JAFFE, H. L. Tumors and Tumorous Conditions of Bones and Joints. Lea & Febiger, Philadelphia, 1958, 629 pp.

25. KOLETSKY, S., and GUSTAFSON, G. E. Whole-body radiation as a carcinogenic agent. *Cancer Res.*, 1955, **15**, 100–104.

26. WARREN, S. Effects of radiation on normal tissues. XIII. Effects on the skin. *Arch. Path.*, 1943, **35**, 340–347.

27. JONES, A. Irradiation sarcoma. *Brit. J. Radiol.*, 1953, **26**, 273–284.

28. ANDERSEN, V. Roentgen sarcoma; report of a case. *Acta radiol.*, 1956, **45**, 155–160.

29. RÉCAMIER, D., and TRIBONDEAU, L. À propos de l'action des rayons X sur l'ostéogenèse. *Compt. rend. Soc. biol.*, 1905, **59**, 621–622.

30. EWING, J. Radiation osteitis. *Acta radiol.*, 1926, **6**, 399–412.

31. GALL, E. A.; LINGLEY, J. R., and HILCKEN, J. A. Comparative experimental studies of 200 kilovolt and 1000 kilovolt roentgen rays. I. The biological effects on the epiphysis of the albino rat. *Am. J. Path.*, 1940, 16, 605–618.

32. SISSONS, H. A. Experimental Study of the Effect of Local Irradiation on Bone Growth. In: Progress in Radiobiology. Oliver & Boyd, London, 1956, pp. 436–448.

33. KOHN, H. I., and KALLMAN, R. F. Acute lethality studies with the rat: the LD ∞, death rate, and recovery rate. *Radiation Res.*, 1957, 7, 85–97.

The investigation was begun while one of us (H.L.) was working in the Department of Radiotherapeutics of the University of Cambridge under a special grant from the British Empire Cancer Campaign which is gratefully acknowledged. One of us (D.B.C.) held a British Empire Cancer Campaign Research Fellowship during the period of the investigation. The third author (R.B.) is a United States Public Health Service Senior Research Fellow.

Our grateful thanks go to Miss N. C. Burr of the Department of Pathology, University of Cambridge, England, for her technical assistance.

LEGENDS FOR FIGURES

Photomicrographs were prepared from sections stained by hematoxylin and eosin.

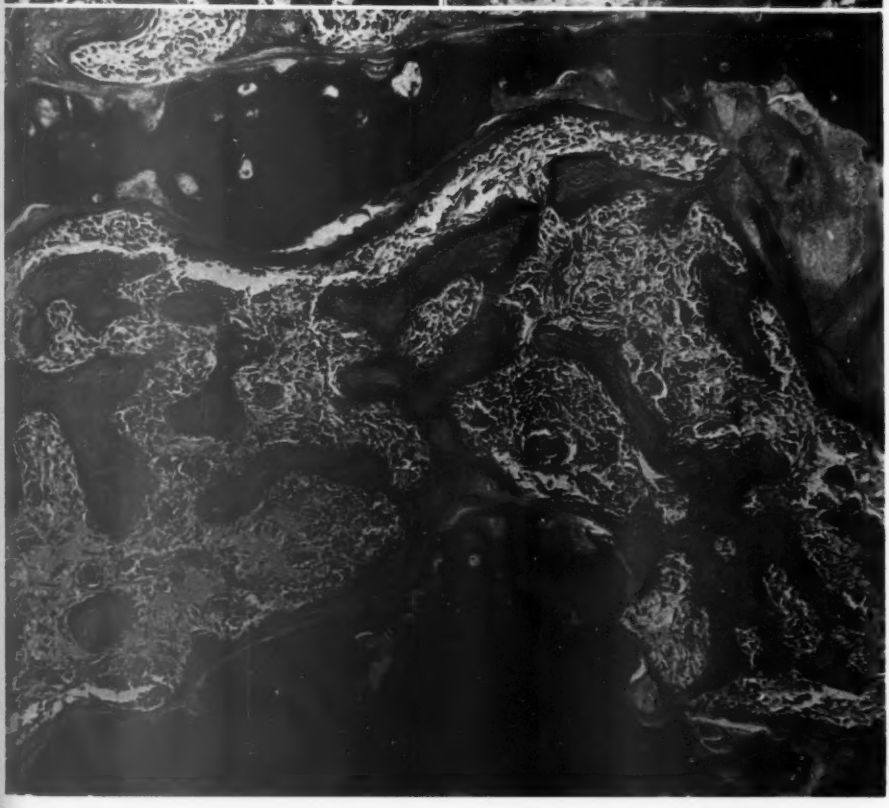
FIG. 1. Epiphysial cartilage in the tibia of a 600-day-old control rat. The field includes the proximal epiphysial plate and part of the metaphysis. × 170.

FIG. 2. A severely damaged cartilage plate in the proximal tibial epiphysis. Female rat, 689 days after local irradiation. × 170.

FIG. 3. Doubling of the epiphysial plate in the tibia. Rat, 499 days after irradiation. × 224.

1

2



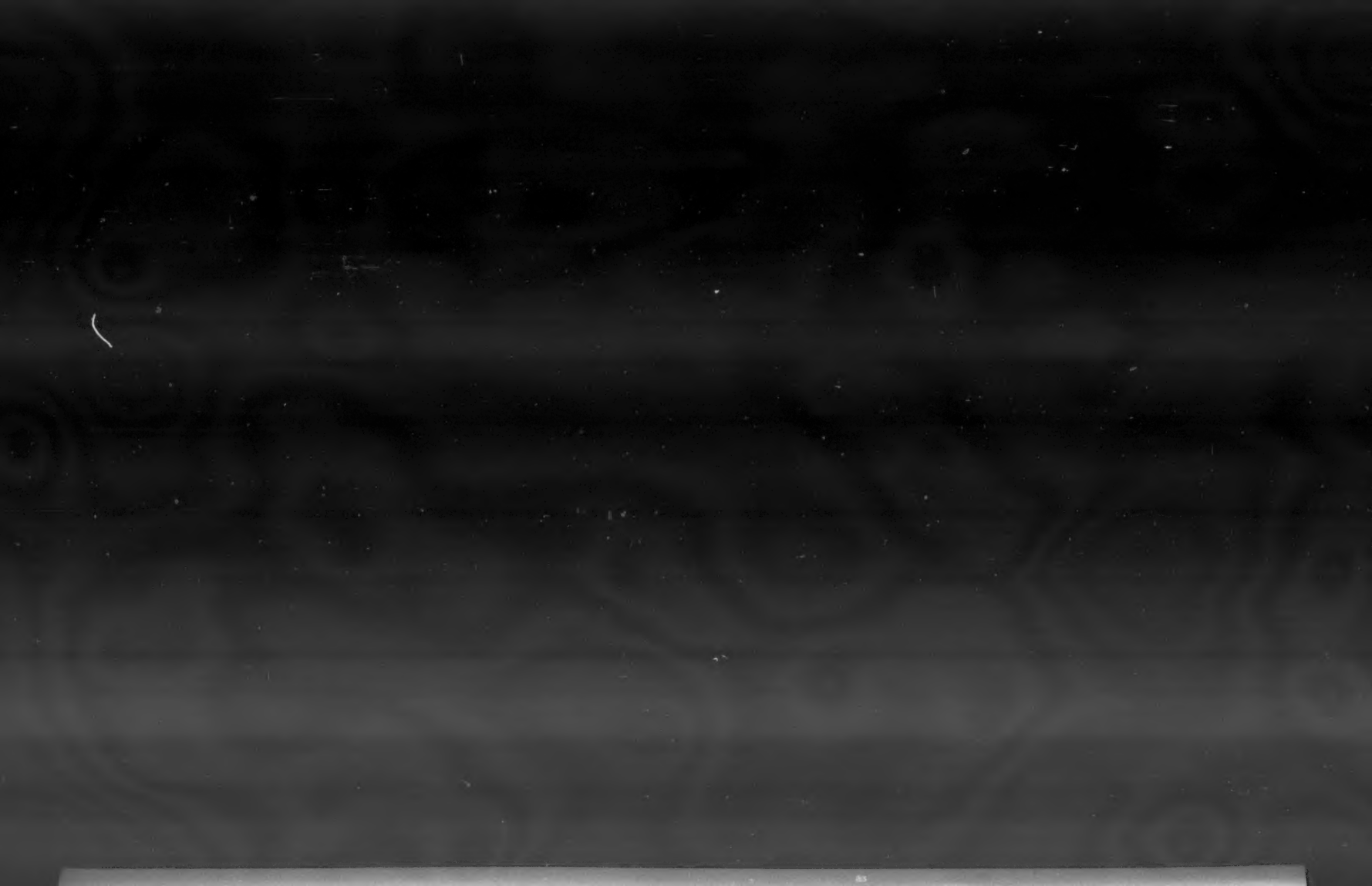
3

FIG. 4. Femur of a female rat, 461 days after local irradiation. Devitalized spongiosa appears at the mid-shaft, and there is newly formed, unresorbed spongiosa in the metaphysis. × 7.5.

FIG. 5. Femur of a female rat, 612 days after irradiation. A partially resorbed devitalized spongiosa lies at mid-shaft, and a small nest of atypical bone may be seen beneath the thickened cartilage plate. × 7.5.

FIG. 6. Fibrous marrow spaces in the tibial metaphysis. Female rat, 554 days after local irradiation. × 99.

FIG. 7. Bone cysts in the femur of a rat, 516 days after local irradiation. There is atypical bone in the epiphysis. × 7.5.



4

5

6

7

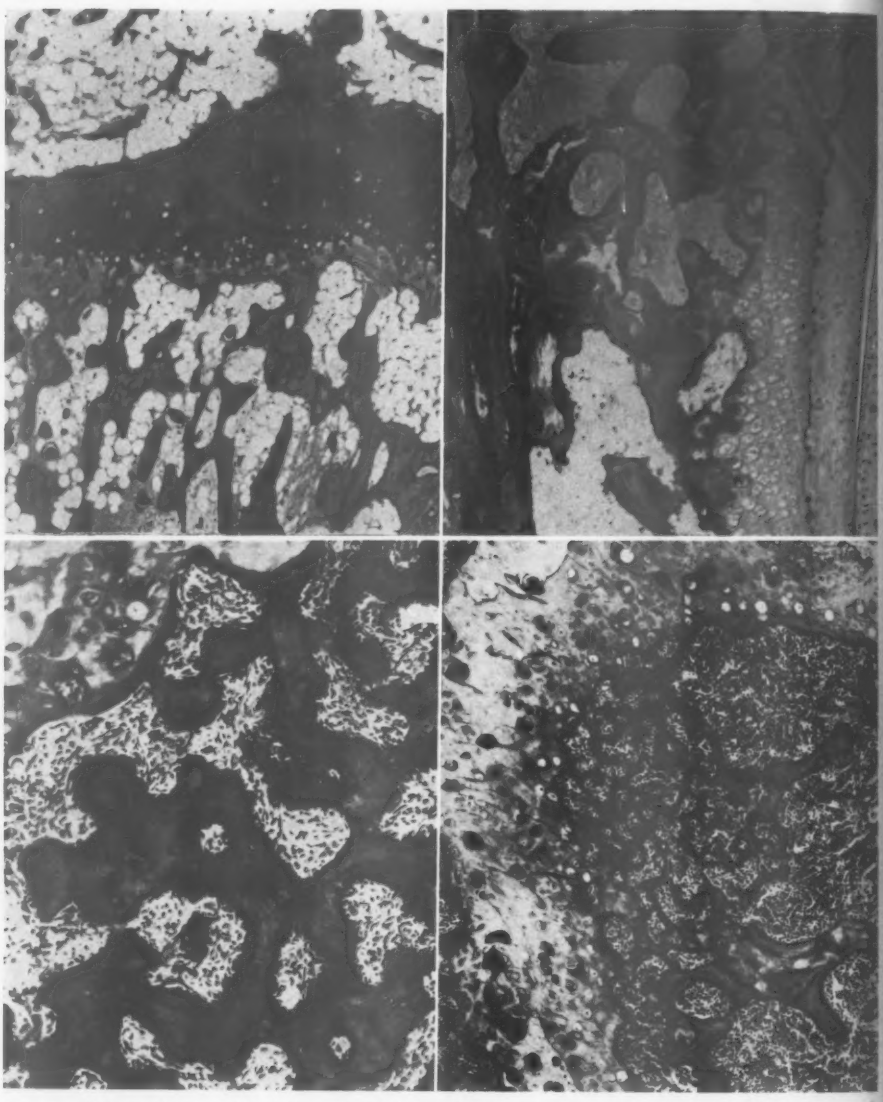
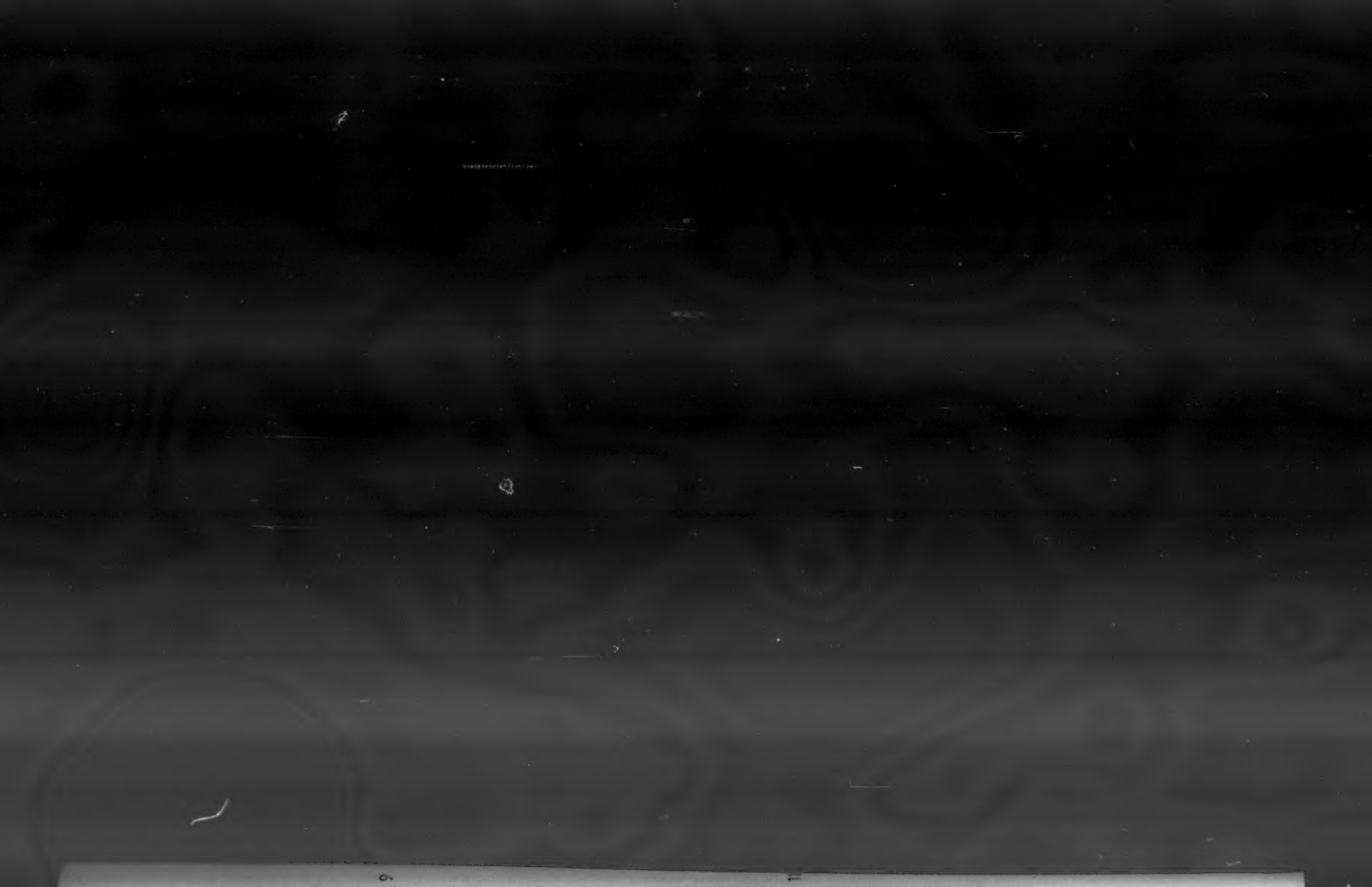


FIG. 8. Fibrous bone, thickened cartilage plate and fatty marrow are evident in the tibia of a rat, 388 days after local irradiation. × 99.

FIG. 9. Fibrous bone and fibrous marrow spaces are manifest in the patella of a rat, 516 days after local irradiation. × 60.

FIG. 10. Atypical bone may be seen in the tibial metaphysis of a rat, 644 days after local irradiation. × 170.

FIG. 11. A nest of atypical bone lies beneath the cartilage plate in the femur of a rat, 612 days after irradiation. This is a higher magnification of the lesion shown in Figure 5. × 60.



STUDY ON THE HEALING OF CARDIAC NECROSIS IN THE RAT

GEORGE RONA, M.D.; DAVID S. KAHN, M.D., AND CLIFFORD I. CHAPPEL, PH.D.

From Ayerst Research Laboratories, the Department of Pathology, St. Mary's Memorial Hospital, and the Department of Pathology, McGill University, Montreal, Canada

It has been reported earlier that rats receiving subcutaneous injections of 1-(3,4-dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride (isoproterenol) on two consecutive days will develop myocardial necrosis. This depends on the size of the dose and the weight of the animal.[1,2] We have used this observation to develop a pharmacologic technique for producing myocardial necrosis of standard severity in animals. The myocardial necrosis was thought to result from a relative ischemia of the heart muscle. It was postulated that this was due to a combination of increased cardiac oxygen requirement as a result of the cardiac stimulant effect of the drug and a reduction of coronary blood flow secondary to a decrease in the systemic blood pressure.[3] Following the acute phase, the circulation appeared to return to normal, and at necropsy no lesions of the coronary arteries were demonstrable.[4] Previous reports have described only the acute lesions produced in the myocardium.[5] However, the technique also offers an opportunity to study the healing of myocardial necrosis in hearts with a normal blood supply.

The present report deals with the healing of this type of experimental myocardial necrosis in animals observed up to 1½ years after injury. A comparison will also be made with the observations of other investigators who studied infarction induced by occluding a major coronary artery[6,7] and other experimental myocardial lesions.[8]

MATERIAL AND METHODS

One hundred twenty male Wistar rats, weighing an average of 205 ± 4 gm., were used. They were housed at a temperature of 75 ± 2° F. and a relative humidity of 45 ± 5 per cent. The diet was Purina rat food. Racemic isoproterenol hydrochloride was injected subcutaneously into 100 rats; the dose was 85 mg. per kg. of body weight in a volume of 1 ml. of aqueous solution. The rats were killed in groups of 5 at 30 minutes, 2, 8, 24, 36, 48 hours, 3, 5, 6, 7 days, and 2, 3, 5, 8, 10, 12, 16, 32, 52 weeks, and 1½ years after the first injection. Rats killed after 30 minutes, 2, 8 and 24 hours received only one injection of isoproterenol; all the others received injections on two consecutive days. Control groups of 5 rats each were given injections of 1 ml. of distilled water on two consecutive days, and killed 48 hours, 8 weeks, 1 year, and 1½ years after the first injection.

The symptoms and mortality of the rats were recorded and necropsies were performed. The gross lesions of the heart were graded according to a modification of the

Accepted for publication, May 23, 1961.

system described in detail elsewhere.[5] This may be summarized as follows: grade 0, no lesions; grade 1, mottling of the apex and distal parts of the left ventricle caused by intermingled pale and dark red streaks; grade 2, well-demarcated necrotic areas or scars limited to the apex; grade 3, large infarct-like foci of necrosis or scars involving at least one third of the left ventricle and extending to adjacent areas of the right ventricle and interventricular septum; grade 4, large infarct-like areas of necrosis or acute or chronic aneurysm formation.

After fixation in Bouin's fluid, frontal sections were made of the heart. These included both atriums and ventricles and the interventricular septum. Cardiac blocks and those from other organs were embedded in paraffin; sections were cut at 5 μ and were stained with hematoxylin and eosin, Cason's trichrome stain,[9] the periodic acid-Schiff (PAS) reaction,[10] and occasionally with phosphotungstic acid-hematoxylin (PTAH),[11] Foot's silver impregnation,[12] and the colloidal iron stain.[13]

Results

Clinical Symptoms and Mortality

The rats showed characteristic symptoms within 20 minutes of treatment. They assumed a typical posture, lying on their sides or backs, with the head extended, and they breathed with mouth open. Heart beats were strong at first and later weak and very rapid. Respiration became irregular and of abdominal type. The symptoms decreased after 6 to 8 hours, but for 24 hours the animals were weak and immobile as though sedated. There was cyanosis of the mucous membranes, edema of the tongue, and a bloody discharge from the nostrils of some animals. These signs and symptoms were accentuated after the second injection, which caused the death of 5 rats. These 5 form the 36-hour group. A number of rats also developed swelling of the face and legs, which disappeared 2 to 3 days later. One week after the first injection of isoproterenol the animals appeared completely healthy. No further spontaneous mortality was observed except in 2 rats; one of these died in the 29th and one in the 64th week of the observation period. The remainder showed no clinical abnormalities and gained weight normally.

Necropsy Findings

Rats killed at various intervals within the first 48 hours showed progressively more severe congestion of the liver; less frequently there was congestion of the other abdominal viscera and meninges. All animals which died at 36 hours and 3 of the 5 rats killed 48 hours after the first injection had lung edema and hydrothorax. In the groups killed on the third, fifth, sixth and seventh days, 2, 4, 1 and 3 rats, respectively, had marked congestion and hypostasis of the lungs. One rat killed on the third day and another on the sixth day had multiple hemorrhagic infarcts of the lung. No gross lesion outside the heart was observed in rats killed 2 weeks after injection of isoproterenol except as follows: 1 rat sacrificed in the third week had massive cerebral hemorrhage; 1 rat out of 4 killed

in the 32nd week and 2 other rats, sacrificed 1 year and 1½ years after injury, had liver alterations consistent with cardiac fibrosis. The rats that died spontaneously at 29 and 64 weeks had marked congestive cardiac failure, characterized by anasarca, hydrothorax, and a liver lesion consistent with cardiac fibrosis.

Gross Examination of the Heart

The hearts of rats killed one half and two hours after the first treatment were dilated. Later the ratio of heart weight to body weight showed a progressive increase and was significant at the 99 per cent level of confidence after 8 hours (Table I). The peak of the enlargement was

TABLE I

CARDIAC LESIONS FOLLOWING ISOPROTERENOL INJECTION *

Time of autopsy	Average heart wt. (mg./100 gm. body wt.)	Gross heart lesion; average severity	Aneurysm
30 min.	361 ± 13 †	0	0
2 hr.	398 ± 27	0	0
8 hr.	441 ± 48	2.0	0
24 hr.	541 ± 23	3.0	0
36 hr.	627 ± 32	3.6	3
48 hr.	654 ± 23	3.6	3
	343 ± 6 ‡		
3 days	544 ± 16	3.6	3
5 days	523 ± 53	3.0	2
6 days	433 ± 12	2.8	1
7 days	443 ± 22	3.0	2
2 wk.	373 ± 9	2.6	1
3 wk.	372 ± 19	2.0	0
5 wk.	324 ± 17	1.0	0
8 wk.	340 ± 9	2.4	2
	307 ± 5 ‡		
10 wk.	312 ± 20	2.0	0
12 wk.	323 ± 13	3.0	2
16 wk.	346 ± 16	2.8	1
32 wk.	372 ± 5	3.0	2
1 yr.	390 ± 22	3.0	3
	303 ± 10 ‡		
1½ yr.	368 ± 19	3.0	3
	285 ± 10 ‡		

* Each period cited indicates a group of 5 rats given isoproterenol subcutaneously (85 mg. per kg. of body weight); those sacrificed at 30 minutes to 24 hours received only one injection; all others received injections on 2 consecutive days.

† Standard error of the mean.

‡ Values of nontreated animals.

48 hours after the first treatment; thereafter, the heart weights declined but were still significantly enlarged one week after the first injection. The relative heart weights were within normal limits in rats killed at two weeks. After 16 weeks the relative heart weights showed a tendency to

increase; the enlargement of the heart at this time was related to chronic aneurysm formation seen in a number of the animals (Fig. 1). Where control data were available, a significant difference existed between control animals and those given isoproterenol.

No gross lesion was observed in the heart until 8 hours, when indistinct pale or mottled subendocardial, mostly apical, lesions were seen. All the rats killed at 24 hours or later had well-marked gross cardiac necrosis; some of them also had acute aneurysms. The average grade of severity of the gross cardiac necrosis observed in the various groups is indicated in Table I. In the second week the yellow color in the areas of infarction changed to a grayish white, and the lesions became firm and elastic. Later the extent of the lesions diminished and in some cases they became indistinct or not visible. Some of the rats, however, developed chronic aneurysms of the apical portion of the heart, occasionally involving both right and left ventricles. There was a marked deformity of the heart. Ventricles were enormously dilated and were paper-like in thickness. In rats killed later than 16 weeks, there was fibrous thickening of the apical epicardium with the formation of firm, grayish white round or oval plaques measuring from 0.3 to 1 cm. in diameter.

Microscopic Examination of the Heart

Thirty minutes after injection, the structure of the myocardium was generally preserved. Some subendocardial fibers in the apex of the left ventricle contained short segments which stained intensely with eosin and had a weak PAS reaction. Gross striations of the affected muscle fibers were difficult to find even in PTAH-stained sections. The capillaries bordering these segments showed some dilatation. There was a quantitative increase in eosinophilia of the muscle fibers 2 hours after injection. These fibers were generally located near dilated capillaries. Occasional arterioles had a prominent mantle of adventitial cells. A few small epicardial collections of mast cells, histiocytes and lymphocytes were found. Eight hours after isoproterenol administration large groups of myocardial fibers showed deep eosinophilic staining and intensive PAS reaction. The homogeneous fibers stained bright red with Cason's trichrome. Short segments of fibers were anuclear and a very occasional fiber segment exhibited granular disintegration. The adjacent capillaries were widely dilated and filled with neutrophils. Here and there, fibers were separated, indicating interstitial edema. In the ventricular subendocardiums a few small collections of neutrophils appeared.

Twenty-four hours after the injection, focal infarction was quite evident. Capillaries were widely dilated and filled with neutrophils, and interstitial edema was more pronounced. Neutrophils appeared in proc-

ess of migration into the interstitial tissue. Arteriolar adventitial proliferation was distinct. Focal cellular infiltration was manifest in the auricular epicardium. The myocardial lesions were qualitatively similar but more diffuse 36 hours after isoproterenol treatment (Fig. 2). In addition to the reaction described, there were scattered large cells with pale swollen nuclei. They were located in the subendocardial and perivascular regions and were interspersed with collections of large cells with the cytologic characteristics of histiocytes. Forty-eight hours after injection, the myocardial necrosis was extensive (Fig. 3), but never completely homogeneous since viable areas alternated with completely necrotic ones. Capillary dilatation was less striking, but separation of fibers by edema fluid was still quite marked. Exudate, though still present, was composed predominantly of large, mononuclear histiocytes.

Seventy-two hours after injection, regression of the acute myocardial lesions was evident. Most of the necrotic fibers had disappeared, and only a few PAS-positive homogeneous fibers remained. An interstitial reaction consisted of mild edema, a large number of fusiform mononuclear cells and Anitschkow myocytes and some young fibroblasts (Fig. 4). In both ventricles a few large areas were completely replaced by this proliferative reaction, and many focal granuloma-like lesions of this type could be seen in the subendocardial region. At 5 days only a few neutrophils remained, and it was very difficult to find residual necrotic muscle fibers. The inflammatory reaction was very sharply outlined; some of the reactive cells were plump and fusiform, resembling fibroblasts, and scattered reticulin fibers were recognized in the interstitial lesion. On the sixth and seventh days the predominant pattern was one of early repair. Cellular lesions were composed of elongated fusiform cells and Anitschkow myocytes; the proliferation of these cells tended to be both focal and diffuse (Figs. 5 and 6). The reaction was most frequent in the subendocardial layer and in the perivascular connective tissue. In 2 of 10 rats examined at this stage, fibroblastic and Anitschkow cell proliferation was evident near the attachment of the mitral valve cusps with slight piling up of endothelial nuclei.

In the groups sacrificed 2, 3 and 5 weeks after isoproterenol administration, necrotic and exudative alterations were almost completely absent. There was early focal fibrous scarring, with moderate numbers of spindle cells and a few pigment-containing macrophages and lymphocytes. An occasional epicardial scar was noted. In two cases there were small granulomas and vascularized scars at the bases of the mitral valves (Fig. 7). One animal showed a small verrucous scar on the undersurface of the mitral valve.

In animals sacrificed at 8, 10 and 12 weeks, there were variations in

the severity of scar formation. In two rats with macroscopic aneurysms, dense scar replaced the apical portions of the ventricles. Other rats had focal myocardial scar formation. Only occasional inflammatory cells appeared in the scar tissue.

Three of the 10 rats sacrificed at 16 and 32 weeks had aneurysmal dilation of the left ventricle; in these the sacs were composed completely of connective tissue. In 2, cartilage and bone had formed in the aneurysmal wall. The hearts with aneurysms revealed disseminated, loosely cellular foci containing lymphocytes and large mononuclear cells. These were particularly pronounced at the transition zone between scar tissue and adjacent viable muscle fibers. In other rats there were merely scattered, dense, myocardial scars containing a few lymphocytes and histiocytes.

Rats killed at 1 and 1½ years showed similar myocardial alterations, and the two groups are discussed together. Six of the 10 rats had large ventricular aneurysms; the apical portion of both ventricles and of the interventricular septum was replaced by dense scar tissue. In 5 aneurysms focal osseous or cartilaginous metaplasia was observed (Fig. 8); the bone lamellas lay parallel to the endocardial surface. In 2 aneurysms the scar tissue was separated by fat into external (pericardial) and internal (endocardial) layers. In the scars, perivascular accumulations of swollen histiocytes were focally apparent. Focally perivascular and subendocardial fibrosis were encountered; fibroblasts were swollen here, and there was proliferation of Anitschkow myocytes. Several well-circumscribed foci contained similar cells (Fig. 9) and also an occasional multinucleated cell resembling the Aschoff giant cell. These lesions exhibited a perivascular or subendocardial predilection. An increased prominence of capillaries was observed, some showing cavernous dilatation and engorgement. In occasional arterioles there were swollen endothelial cells, thickened walls, reduced lumens and prominent mantles of adventitial histiocytes. In the rats lacking aneurysms, there were dense linear or plexiform scars separating individual muscle fibers or bundles of them from each other. The scars, when stained by the colloidal iron method, contained considerable numbers of thin, curled, collagen fibers (Fig. 10), and silver impregnation revealed fine, delicate argentaffin fibers. Muscle fibers adjacent to scars were markedly enlarged and exhibited vacuolar swelling and irregular hyperchromatic nuclei. Collections of lymphocytes and histiocytes were observed occasionally in the subpericardial fat tissue. Aside from focal sclerosis, no valvular lesions were observed.

Microscopic examination was made of the internal viscera, striated muscle, skin and the knee joint in every animal. Except for the con-

gestive lesions described above, no significant alterations were noted.

Four groups of 5 control rats were sacrificed with the groups necropsied at 48 hours, 8 weeks, 1 year and 1½ years after isoproterenol injection. Except for occasional fibrotic foci in the atrioventricular rings and in the apical portion of the interventricular septum of 2 old rats, no significant histologic changes were detected.

Discussion

Studies of healing in the injury of hearts with intact blood supplies, as in the present experiment, have been limited to myocardial lesions produced by physical methods.[14] The results of such experiments are difficult to evaluate, since the procedures markedly disturbed the function of adjacent structures as well as the heart. Other investigations have been concerned with the development and repair of myocardial necrosis in rats and dogs following ligation of the coronary artery.[6,7] In these animals healing took place in the face of ischemia in the necrotic region, the outcome depending on the availability of a blood supply via collateral channels.[15]

In the present experiment mild alterations developed in the myocardium within 30 minutes of isoproterenol administration, and distinct degeneration was apparent at 2 hours. At 8 hours homogenization of muscle fibers was marked, and these exhibited an intense PAS staining reaction. These observations are in accordance with those reported in rats after coronary artery ligation by Kaufman, Gavan and Hill[16] and by Yokoyama, Jennings, Clabaugh and Wartman.[17] Klionsky[18] demonstrated that this PAS-positive reaction appeared only after the initial loss of glycogen from the fibers. In our isoproterenol experiments, myocardial necrosis was prominent at 24 hours but was not of homogeneous nature. Completely necrotic bundles alternated with others less severely affected; this was similar to the lesion produced in dogs by coronary artery ligation.[19] The patchy necrosis presumably occurred in those areas with the most severe ischemia during the period of myocardial stimulation.

Capillary engorgement was pronounced 1½ days after injury, and a marked cellular exudate and edema separated the necrotic muscle fibers. Tenant, Grayzel, Sutherland and Stringer[20] and Bronson[21] have reported a similar marked alteration of capillary permeability and excessive exudation when, after a short period of coronary artery occlusion, the circulation was restored. The ensuing hemorrhagic and exudative type of response differed from the regressive myocardial lesions found in permanent arterial occlusion. The abundant exudative response in the present experiment probably reflected the restoration of coronary circu-

lation through damaged capillaries and myocardium following the initial period of anoxia.

The peak of regressive alterations and the beginning of fibroplasia were found 2 days after isoproterenol injection. The early proliferation of fibroblasts paralleled that observed in the healing of myocardial infarction in the dog [7]; it was at variance with the findings in human subjects [22] in whom fibroblastic proliferation occurs at a later time. Proliferation of Anitschkow myocytes was observed in the vicinity of blood vessels or beneath the endocardium, and the association of these cells with adventitial and subendocardial histiocytes suggested an interrelationship. In contrast to coronary artery ligation in the dog, in which necrotic muscle remained for weeks, in the present experiment necrotic fibers were almost completely replaced by a granuloma-like reaction by 3 to 5 days after injury. The cells comprising the lesion were young fibroblasts, histiocytes, and a few multinucleated giant cells, resembling Aschoff cells. After a week the granulomatous reaction exhibited fibrous replacement.

The pathogenesis of the granuloma-like reaction requires explanation. Similar lesions have not been reported in rats with coronary artery ligation or in those with myocardial infarction following experimental coronary atherosclerosis.[23–26] In the latter group, however, detailed studies of the myocardial lesion were not recorded since attention was focused on the vascular alterations. Karsner and Dwyer,[7] in their study of the healing of myocardial infarction in dogs, observed occasional myocytes and foreign body giant cells at the edges of the healing infarcts 16 days after coronary artery ligation, but reported no inflammatory reaction after 10 weeks. Focal myocardial necrosis with a granulomatous reaction similar to that noted in the present experiment has been produced in the rabbit by a number of investigators. Murphy and Swift [27] used multiple subcutaneous injections of group A streptococci; Kellner and Robertson [28] introduced proteolytic enzymes intravenously; Geschickter, Athanasiadou and O'Malley [29] used dimethylphenylenediamine, a strong reducing agent; and More and Movat [30] gave multiple injections of horse serum. In these animals, as in the present experiment, normal coronary circulation was presumably maintained.

The rat is prone to develop a granulomatous type of reaction in the healing of many damaged tissues.[31] However, the fact that other reports [6,32] on the healing of myocardial infarction produced in rats by permanent coronary artery occlusion do not mention this type of reaction suggests that it is related to some feature in the present experiment. Two factors may be of significance. A pharmacologic action of isoproterenol may constitute one of these. There is no information in this regard except that a similar reaction was not induced in other tissues.

A second, more likely factor is the retention of a normal blood supply to the injured area during the healing process.

The early granuloma-like lesions bear some histologic similarity to Aschoff nodules as observed in rheumatic fever. According to Saphir,[33] the Aschoff nodule is a characteristic microscopic structure that occurs only in the acute granulomatous stage of human rheumatic fever. Other investigators disagree, however, and feel that close histologic resemblances in experimental and human lesions allow varied inferential deductions to be drawn regarding the pathogenesis of rheumatic cardiac lesions.[8] In the present study granulomas developed about degenerating muscle fibers. These resembled the "myofiber" Aschoff body described by Murphy,[34] who suggested that the multinucleated giant cells were myogenic in origin.[35] Since some of the "granulomas" in the present experiment seemed to occur independently of degenerating muscle, there is no certainty of their origin, except that they undoubtedly represent secondary consequences of the primary myocardial damage.

In addition to the granuloma-like lesions found during the first few weeks of the present experiment, small "granulomas" were found at intervals up to 1½ years in animals that developed aneurysms. These could represent the result of repeated minor mechanical injuries secondary to alteration in tension forces, a consequence of aneurysm suggested by Murray.[36] Damaged fibers might thus incite focal granulomatous reactions. Whether this was the mode of development or whether the lesions were merely residua from the early necrotic stage could not be established. One may raise the query as to whether focal inflammatory lesions found in chronic rheumatic heart disease necessarily indicate active rheumatic disease. Lesions of this nature might conceivably be provoked by some mechanical disturbance of cardiac contractility, a situation for which there is a good morphologic basis in chronic rheumatic heart disease. It might, moreover, explain the discrepancy between the clinical assessment of rheumatic activity and the morphologic features encountered in resected atrial appendages.[37,38]

Further points of interest in this study were the two distinct sequels of myocardial infarction. In the early stages the myocardial abnormalities appeared to be diffusely distributed throughout the ventricles. On the other hand, rats sacrificed late in the study exhibited two distinct patterns. In one, the heart was of normal weight and only thin linear scars were distributed throughout the myocardium. In a second group the hearts were markedly enlarged, large aneurysms were manifest, and microscopically there was widespread replacement of the muscle by scar tissue. These two healing patterns suggest that some factor in addition to the extent of the original necrosis played a role.

Gross and Steinberg[39] have classified the mechanisms of myocardial

infarction occurring in man without occlusive coronary lesions. Except for a subendocardial localization of necrosis, Friedberg and Horn[40] found no pathologic distinction between this type of lesion and those resulting from coronary occlusion. We have found no studies concerned with the healing of such non-occlusive lesions. The miliary infarcts described by Barrie and Urback[41] and the "focal myocytolysis," reported by Schlesinger and Reiner[42] (considered to be a nonspecific response of the myocardium to a variety of toxic injuries in addition to anoxemia) may bear some relationship to the present study. It is indeed possible that some forms of human interstitial myocarditis[43,44] may represent reactions to myofiber injury in hearts with normal blood supplies. If a myocardial reaction similar to that seen in the rat occurs in man, the initial necrotic stage will have passed by the time of necropsy examination.

Summary

Myocardial necrosis of uniform severity was produced in the rat by the subcutaneous administration of isoproterenol (85 mg. per kg. of body weight) on two consecutive days.

The healing of infarct-like myocardial lesions was examined at intervals from 30 minutes to 1½ years. As the cardiotoxic drug administered did not cause a permanent interference with coronary circulation, the technique permitted the investigation of the healing of initially anoxic myocardial damage in the circumstance of normally maintained coronary blood supply.

In the case of regressive myocardial alterations, a reactive granuloma-like response was noted. The histogenesis of these "granulomas" and their possible relationship to similar lesions in the human heart were discussed.

Although in the acute stage the myocardial lesions were quite uniform in severity, rats killed at longer intervals after injury revealed distinctly different healing patterns. In one group of rats the heart lesions healed with a residua of indistinct linear scars; in another group large cardiac aneurysms developed.

Addendum

At the combined meeting of the Canadian Association of Pathologists and the Quebec Association of Laboratory Physicians and Pathologists, June, 1961, Dr. C. P. Handforth of Dalhousie University, Halifax, Nova Scotia, presented a paper entitled "Isoproterenol Induced Myocardial Infarction in Animals," in which he discussed the pathogenesis of isoproterenol heart necrosis. He showed that in hamsters, significant myocardial ischemia occurred 10 minutes after subcutaneous injection of

25 mg. per kg. of isoproterenol. This ischemia affected the inner half of the myocardium of the left ventricle and preceded the necrosis. Similar experiments in rabbits suggested that isoproterenol dilated pre-existing communications between coronary arteries and veins, and caused blood to by-pass the capillary bed in the myocardium. The distribution of the ischemia and the location of the subsequent necrosis could be explained by assuming that the intramural pressure during cardiac systole prevented perfusion of the capillary bed when the by-pass channels were dilated.

According to this theory, isoproterenol heart necrosis is an infarct. The ischemia which causes the infarct is unusual because it is due to the by-pass effect of a vasodilator drug.

References

1. Chappel, C. I.; Rona, G.; Balazs, T., and Gaudry, R. Severe myocardial necrosis produced by isoproterenol in the rat. *Arch. internat. pharmacodyn.*, 1959, **122**, 123–128.
2. Rona, G.; Chappel, C. I.; Balazs, T., and Gaudry, R. The effect of breed, age, and sex on myocardial necrosis produced by isoproterenol in the rat. *J. Gerontol.*, 1959, **14**, 169–173.
3. Chappel, C. I.; Rona, G.; Balazs, T., and Gaudry, R. Comparison of cardiotoxic actions of certain sympathomimetic amines. *Canad. J. Biochem. Physiol.*, 1959, **37**, 35–42.
4. Rona, G.; Zsoter, T.; Chappel, C., and Gaudry, R. Myocardial lesions, circulatory and electrocardiographic changes produced by isoproterenol in the dog. *Rev. canad. biol.*, 1959, **18**, 83–94.
5. Rona, G.; Chappel, C. I.; Balazs, T., and Gaudry, R. An infarct-like myocardial lesion and other toxic manifestations produced by isoproterenol in the rat. *Arch. Path.*, 1959, **67**, 443–455.
6. Johns, T. N. P., and Olson, B. J. Experimental myocardial infarction. I. A method of coronary occlusion in small animals. *Ann. Surg.*, 1954, **140**, 675–682.
7. Karsner, H. T., and Dwyer, J. E. Studies in infarction. IV. Experimental bland infarction of the myocardium, myocardial regeneration and cicatrization. *J. Med. Res.*, 1916, **34**, 21–39.
8. Murphy, G. E. Nature of rheumatic heart disease with special reference to myocardial disease and heart failure. *Medicine*, 1960, **39**, 289–381.
9. Cason, J. E. A rapid one step Mallory-Heidenhain stain for connective tissue. *Stain Technol.*, 1950, **25**, 225–226.
10. McManus, J. F. A. Histological demonstration of mucin after periodic acid. (Letter to the editor) *Nature, London*, 1946, **158**, 202.
11. Lieb, E. Modified phosphotungstic acid-hematoxylin stain. *Arch. Path.*, 1948, **45**, 559–560.
12. Foot, N. C. A technic for demonstrating reticulum fibers in Zenker-fixed paraffin sections. *J. Lab. & Clin. Med.*, 1924, **9**, 777–781.
13. Rinehart, J. F., and Abul-Haj, S. K. An improved method for histologic demonstration of acid mucopolysaccharides in tissues. *Arch. Path.*, 1951, **52**, 189–194.

14. TAYLOR, C. B.; DAVIS, C. B., JR.; VAWTER, G. F., and HASS, G. M. Controlled myocardial injury produced by a hypothermal method. *Circulation*, 1951, **3**, 239–253.

15. JENNINGS, R. B., and WARTMAN, W. B. Reactions of the myocardium to obstruction of the coronary arteries. *M. Clin. North America*, 1957, **41**, 3–15.

16. KAUFMAN, N.; GAVAN, T. L., and HILL, R. W. Experimental myocardial infarction in the rat. *Arch. Path.*, 1959, **67**, 482–488.

17. YOKOYAMA, H. O.; JENNINGS, R. B.; CLABAUGH, G. F., and WARTMAN, W. B. Histochemical studies of early experimental myocardial infarction. Periodic acid-Schiff method. *Arch. Path.*, 1955, **59**, 347–354.

18. KLIONSKY, B. Myocardial ischemia and early infarction. A histochemical study. *Am. J. Path.*, 1960, **36**, 575–591.

19. JENNINGS, R. B., and WARTMAN, W. B. Production of an area of homogeneous myocardial infarction in the dog. *Arch. Path.*, 1957, **63**, 580–585.

20. TENNANT, R.; GRAYZEL, D. M.; SUTHERLAND, F. A., and STRINGER, S. W. Studies on experimental coronary occlusion. Chemical and anatomical changes in the myocardium after coronary ligation. *Am. Heart J.*, 1936, **12**, 168–173.

21. BRONSON, L. H. Anatomical and chemical changes in the myocardium following short term coronary artery occlusion in dogs. *Yale J. Biol. & Med.*, 1937–1938, **10**, 405–410.

22. MALLORY, G. K.; WHITE, P. D., and SALCEDO-SALGAR, J. The speed of healing of myocardial infarction; a study of the pathologic anatomy in 72 cases. *Am. Heart J.*, 1939, **18**, 647–671.

23. WILGRAM, G. F. Dietary method for induction of atherosclerosis, coronary occlusion and myocardial infarcts in rats. *Proc. Soc. Exper. Biol. & Med.*, 1958, **99**, 496–499.

24. THOMAS, W. A., and HARTROFT, W. S. Myocardial infarction in rats fed diets containing high fat, cholesterol, thiouracil, and sodium cholate. *Circulation*, 1959, **19**, 65–72.

25. THOMAS, W. A.; HARTROFT, W. S., and O'NEAL, R. M. Myocardial infarction in man and experimental animals. *Arch. Path.*, 1960, **69**, 104–109.

26. WEXLER, B. C., and MILLER, B. F. Coronary arteriosclerosis and thrombosis in the rat. *Proc. Soc. Exper. Biol. & Med.*, 1959, **100**, 573–576.

27. MURPHY, G. E., and SWIFT, H. F. The induction of rheumatic-like cardiac lesions in rabbits by repeated focal infections with group A streptococci; comparison with the cardiac lesions of serum disease. *J. Exper. Med.*, 1950, **91**, 485–498.

28. KELLNER, A., and ROBERTSON, T. Selective necrosis of cardiac and skeletal muscle induced experimentally by means of proteolytic enzyme solutions given intravenously. *J. Exper. Med.*, 1954, **99**, 387–404.

29. GESCHICKTER, C. F.; ATHANASIADOU, P. A., and O'MALLEY, W. E. The role of mucinolysis in collagen disease. *Am. J. Clin. Path.*, 1958, **30**, 93–111.

30. MORE, R. H., and MOVAT, H. Z. Character and significance of the cellular response in the collagen diseases and experimental hypersensitivity. *Lab. Invest.*, 1959, **8**, 873–889.

31. WILENS, S. L., and SPROULE, E. E. Spontaneous cardiovascular disease in the rat. I. Lesions of the heart. II. Lesions of the vascular system. *Am. J. Path.*, 1938, **14**, 177–216.

32. HEIMBURGER, R. F. Injection into pericardial sac and ligation of coronary artery of the rat. *Arch. Surg.*, 1946, **52**, 677–689.

33. SAPHIR, O. The Aschoff nodule. (Editorial) *Am. J. Clin. Path.*, 1959, **31**, 534–539.

34. MURPHY, G. E. Evidence that Aschoff bodies of rheumatic myocarditis develop from injured myofibers. *J. Exper. Med.*, 1952, **95**, 319–322.

35. MURPHY, G. E. On muscle cells, Aschoff bodies, and cardiac failure in rheumatic heart disease. *Bull. New York Acad. Med.*, 1959, **35**, 619–651.

36. MURRAY, G. The pathophysiology of the cause of death from coronary thrombosis. *Ann. Surg.*, 1947, **126**, 523–534.

37. CLARK, R. M., and ANDERSON, W. Rheumatic activity in auricular appendages removed at mitral valvoplasty. *Am. J. Path.*, 1955, **31**, 809–819.

38. DALLDORF, F. G., and MURPHY, G. E. Relationship of Aschoff bodies in cardiac atrial appendages to the natural history of rheumatic heart disease. *Am. J. Path.*, 1960, **37**, 507–519.

39. GROSS, H., and STERNBERG, W. H. Myocardial infarction without significant lesions of coronary arteries. *Arch. Int. Med.*, 1939, **64**, 249–267.

40. FRIEDBERG, C. K., and HORN, H. Acute myocardial infarction not due to coronary artery occlusion. *J.A.M.A.*, 1939, **112**, 1675–1679.

41. BARRIE, H. J., and URBACK, P. G. The cellular changes in myocardial infarction. *Canad. M.A.J.*, 1957, **77**, 100–106.

42. SCHLESINGER, M. J., and REINER, L. Focal myocytolysis of the heart. *Am. J. Path.*, 1955, **31**, 443–459.

43. MORE, R. H.; MCMILLAN, G. C., and DUFF, G. L. The pathology of sulfonamide allergy in man. *Am. J. Path.*, 1946, **22**, 703–735.

44. SAPHIR, O. Myocarditis in infancy. *Arch. Path.*, 1957, **64**, 446–456.

[*Illustrations follow*]

Legends for Figures

Except where indicated, photomicrographs were prepared from sections stained with hematoxylin and eosin.

Fig. 1. Heart of a rat (right) killed 1½ years after receiving isoproterenol (85 mg. per kg. of body weight) on 2 consecutive days. There is marked enlargement and the formation of an aneurysm involving the apex of the left ventricle. Control heart on the left.

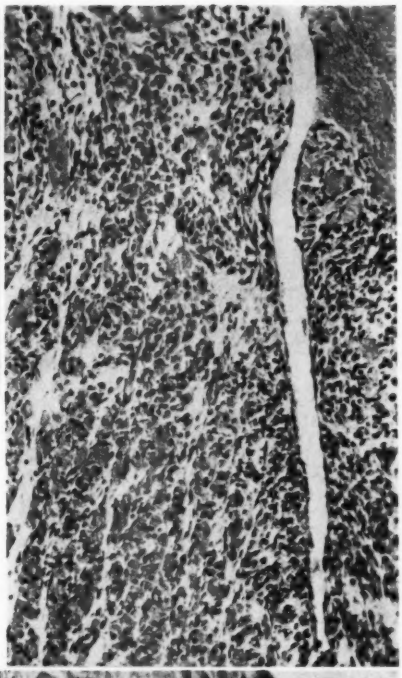
Fig. 2. The apical portion of the left ventricle 36 hours after a subcutaneous injection of isoproterenol. There are diffuse myocardial fiber alterations and marked interstitial edema with early cellular reaction. × 120.

Fig. 3. Confluent infarct-like myocardial necrosis 48 hours after a subcutaneous injection of isoproterenol. Large areas of heart muscle lysis and disorganization of the reticulin framework are manifest. Foot's silver impregnation. × 300.

Fig. 4. Necrotic portions of the heart are replaced by a granuloma-like response 72 hours after injury. × 120.

Fig. 5. A subendocardial granuloma 6 days after isoproterenol injection. The dominant cells are polygonal histiocytes. × 300.

1
2
3


4

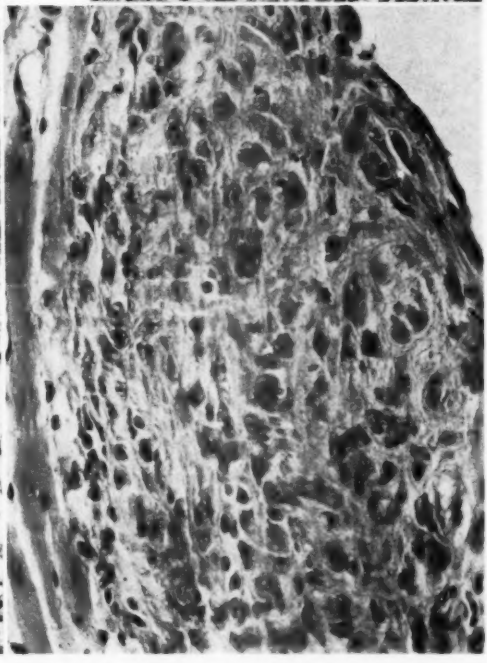

5

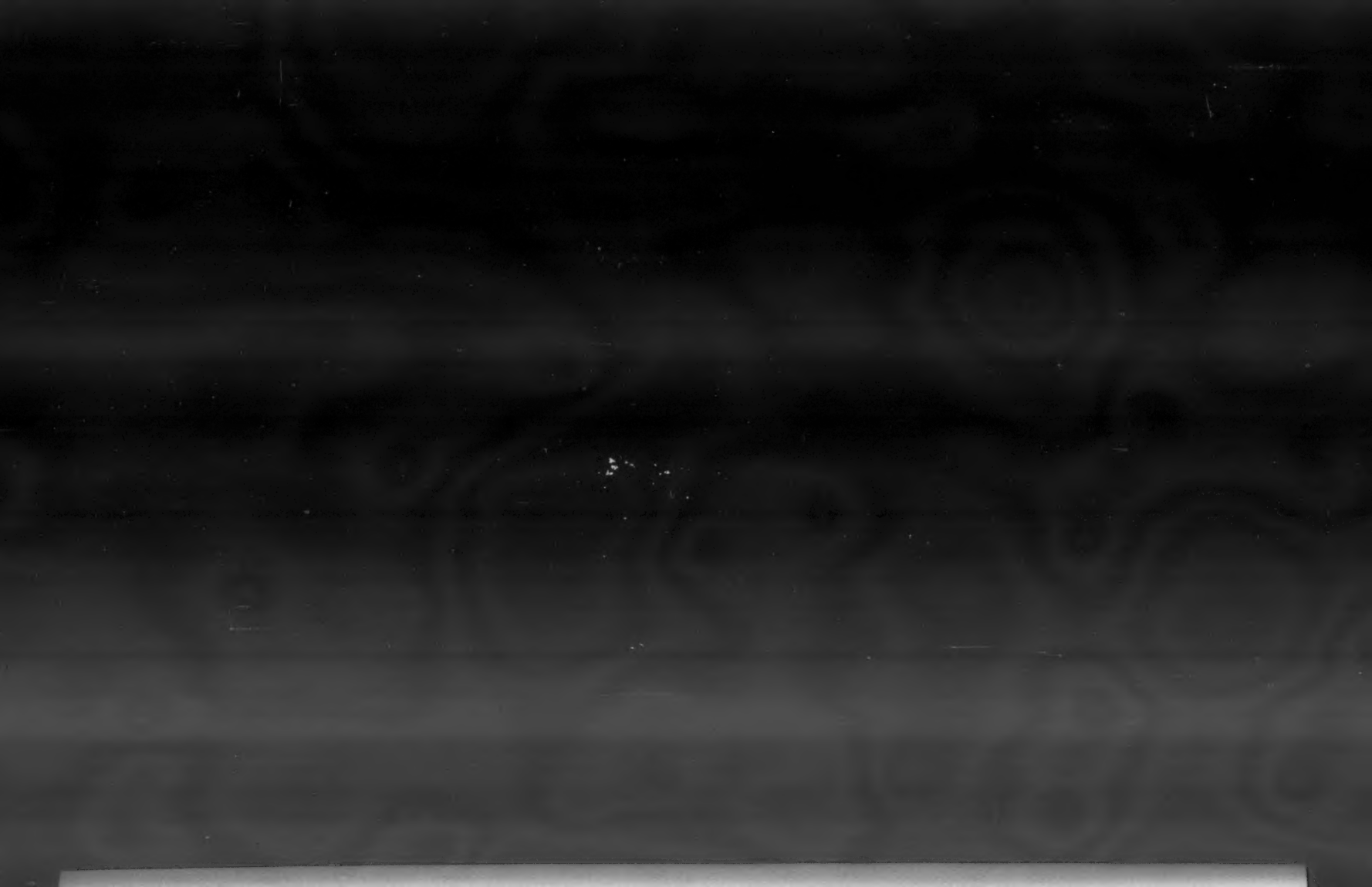
FIG. 6. Diffuse proliferation of fibroblasts and Anitschkow myocytes 7 days after myocardial damage. × 120.

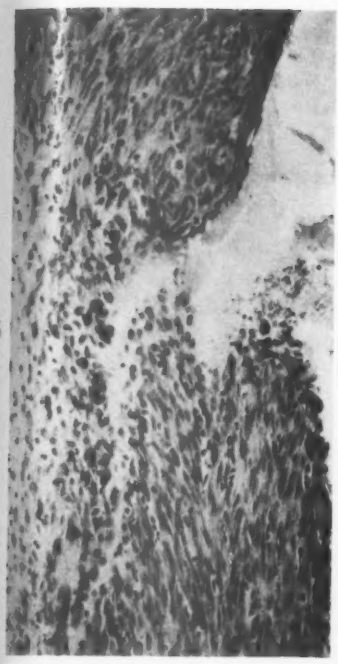
FIG. 7. A granuloma near the base of a mitral valve 3 weeks after myocardial injury. × 300.

FIG. 8. Chronic aneurysm with osseous metaplasia. The rat was killed 1½ years after myocardial injury. Note the marked hypertrophy of the basal portion of the left ventricle. × 8.5.

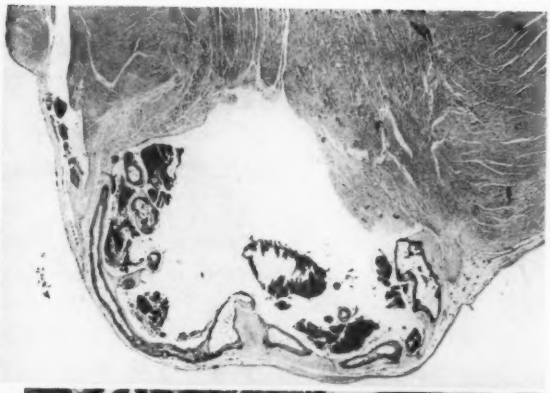
FIG. 9. Subendocardial cellular aggregations in a rat with chronic aneurysm, killed 1½ years after isoproterenol treatment. × 300.

FIG. 10. Collagenization of scar tissue 1½ years after myocardial injury. Colloidal iron stain. × 150.

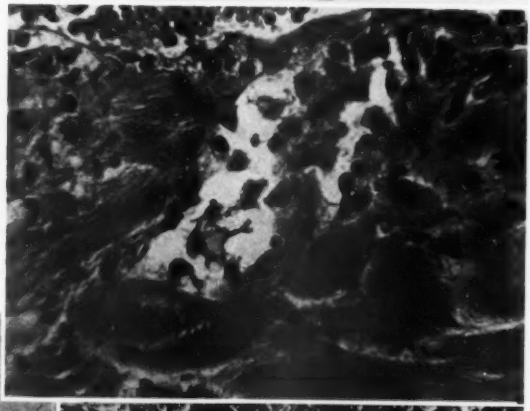




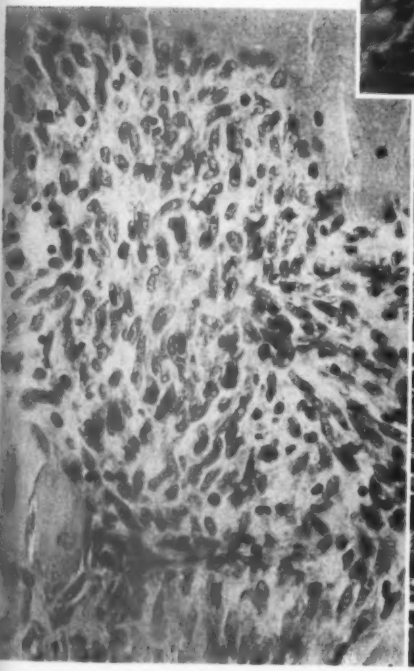
6



8



9



7

10

THE INCIDENCE OF TUMORS AFTER TREATMENT WITH DL-ETHIONINE AND N-2-FLUORENYLACETAMIDE

WALTER R. BENSON, M.D.

From the Department of Pathology, School of Medicine, University of North Carolina, Chapel Hill, N.C.

The effect of the administration of DL-ethionine on the induction of tumors by carcinogenic agents has received scant attention. Miyaji and colleagues [1,2] found that administration of DL-ethionine for periods of 6 to 11 weeks before the administration of dimethylaminobenzene resulted in an acceleration of induction as well as an increase in the incidence of hepatomas in Sprague-Dawley rats. In the present work, the effects of the administration of DL-ethionine on the induction of tumors by N-2-fluorenylacetamide (2FAA) have been studied.

MATERIAL AND METHODS

Female rats of Carworth Farms-Nelson variety, originally a Wistar strain, were used. They weighed from 75 to 115 gm. at the beginning of the study. The animals were housed in separate cages with room temperature being maintained between 75° and 78° F. They remained free of respiratory infections throughout the experiment.

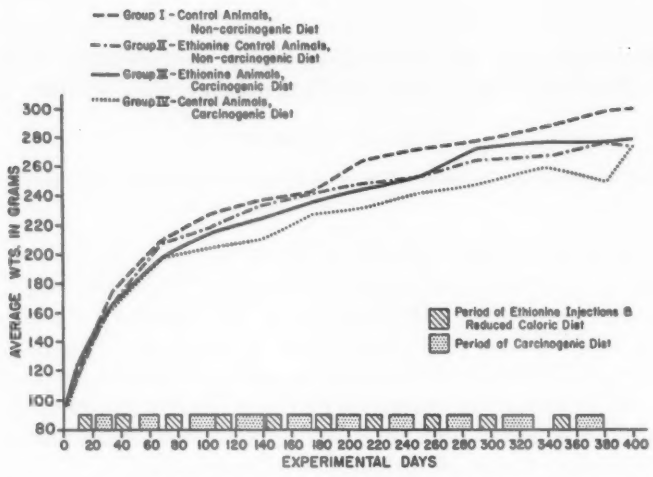
The animals were divided into 4 groups. The 18 animals in group I received intraperitoneal injections of Ringer's solution and the noncarcinogenic food. The 16 animals in group II received intraperitoneal injections of DL-ethionine and the noncarcinogenic food. The 25 animals in group III received injections of DL-ethionine and the carcinogenic food. The 27 animals in group IV received injections of Ringer's solution and the carcinogenic food.

Diet. The major component of the special food was Rockland Farms Chow pellets. The food was prepared as follows: (1) Forty gm. of gelatin were dissolved in 800 ml. of warm water. (2) Ten ml. of 95 per cent alcohol containing 0.2 gm. of 2FAA were added. (3) Four hundred gm. of finely ground Rockland Dog Chow pellets were mixed into the gelatin solution, using a large Waring Blender. The mixture was agitated for 30 seconds, allowed to sit for 1 minute, and then agitated again for 30 seconds. (4) The mixture was poured into shallow glass trays and cooled in a refrigerator. (5) When the mixture was firm, it was cut into cubes and placed in plastic bags, which were sealed and placed in a freezer until needed.

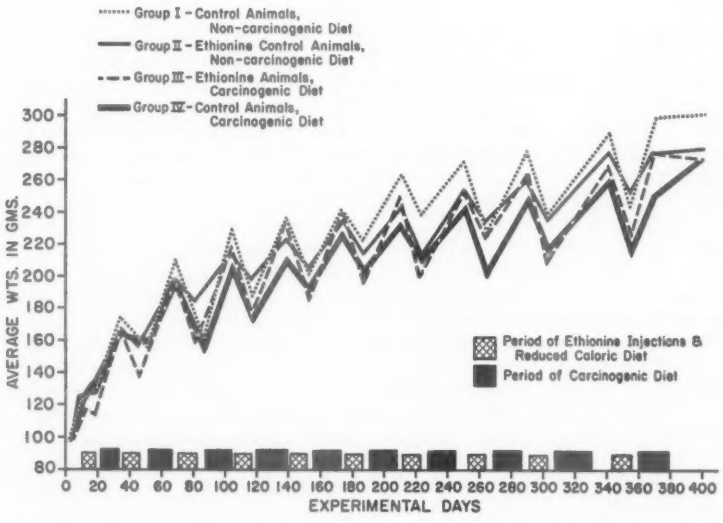
When the carcinogenic diet was administered to the animals in groups III and IV, the animals in groups I and II were given a similarly prepared food without the carcinogen. At all other times the animals were given Rockland Farms Chow pellets. Neither of the gelatin-containing foods was popular. The animals receiving the noncarcinogenic food ate more than those receiving the carcinogenic diet, but none ate as much as they did of the regular pellets. The animals which had been given ethionine injections ate approximately as much food as those which had received Ringer's solution.

This study was supported in part by grants from the National Cancer Institute (C-3339), National Institutes of Health, United States Public Health Service, and the American Cancer Society.

Accepted for publication, April 21, 1961.



TEXT-FIGURE 1. The effect of ethonine or food restriction and the special foods on the gain in weight. The average weights of the animals in each group are shown at the beginning of each period of ethionine injection or food restriction.

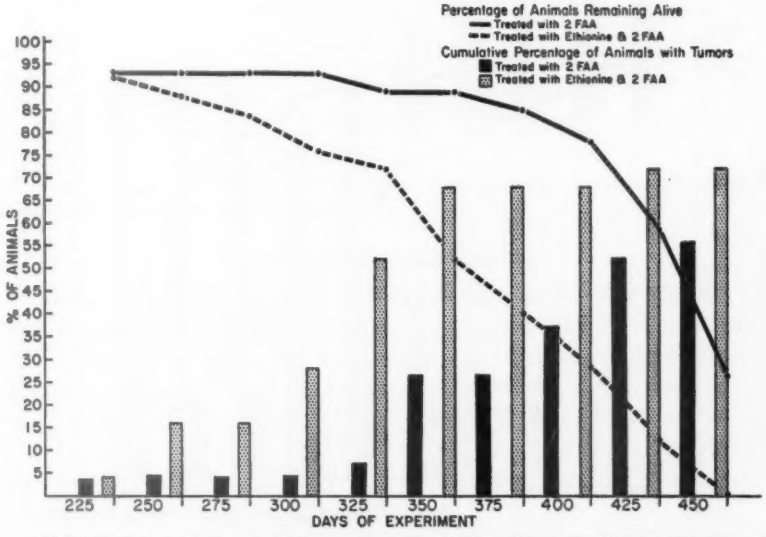


TEXT-FIGURE 2. The losses of weight that occurred during each period of ethionine injection or food restriction and the gains in weight during each period of the special diet. The average weights of animals of each group are shown at the beginning and the end of each period of ethionine injection or food restriction.

Procedure. All animals were given intraperitoneal injections daily for periods of 12 days. The animals in groups II and III received DL-ethionine in sterile Ringer's solution, 25 ml. of DL-ethionine per hundred gm. of body weight. (The ethionine solution was prepared by dissolving 2 gm. of ethionine in 120 ml. of Ringer's solution.) The animals in groups I and IV received injections of similar quantities of Ringer's solution. The majority of those animals receiving ethionine lost considerable amounts of weight (up to 30 per cent). During this time, the amounts of food given to the animals in groups I and IV were reduced so that they lost comparable amounts. The animals were then given a "resting" period of 4 to 6 days. The stock diet was then removed from the cages and the specially prepared foods substituted. For the first 2 to 3 days, the food given to the animals of groups I and IV was restricted to amounts comparable to those consumed by the animals in groups II and III. Afterward they were allowed to eat *ad libitum.* Fresh food was placed in the cages daily. The gelatin foods were given at first for 12 days, then 14, then 18 days, and finally given for periods of 20 or 21 days. At the end of each period, the standard stock diet was again given. The animals were again allowed a rest period of 3 days before the intraperitoneal injections were begun again. Ten such cycles were completed during the study. The animals were weighed and examined daily during the periods of injection. During the other periods, they were examined daily and usually weighed.

Results

The experiment was terminated after 450 days. None of the animals in groups I and II developed tumors, and the majority were permitted to live until the end of the study. The animals in groups III and IV were permitted to live until they became moribund or the tumors bled excessively.



Text-figure 3. The cumulative percentages of the animals developing tumors and the percentage of surviving animals in each group.

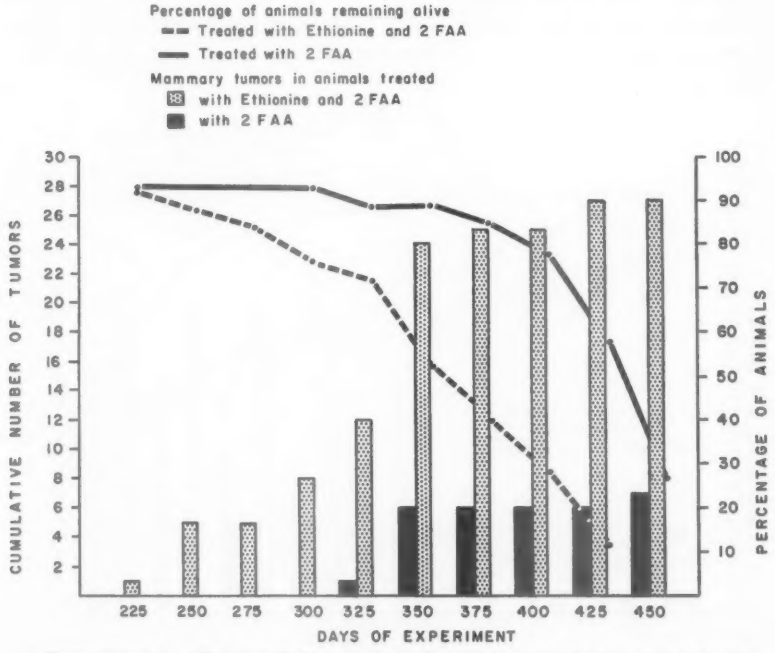
Weight Gains

As indicated in Text-figures 1 and 2, the averages of the weights of the animals in the 4 groups were comparable at the beginnings and the ends of the periods of injection for the first 180 days. After that time, there was a gradually widening gap between the averages of the animals in groups I and IV, although the average weights of the animals in groups III and IV remained within 30 gm. of one another.

Number of Animals Developing Tumors

A larger percentage of animals in group III developed more tumors considerably earlier than did the animals in group IV. By the 375th day of the experiment, 17 of the 25 animals in group III, or 68 per cent, had developed grossly evident tumors. By the same date only 8 of 27 animals in group IV, or 30 per cent, had developed similar tumors (Text-fig. 3).

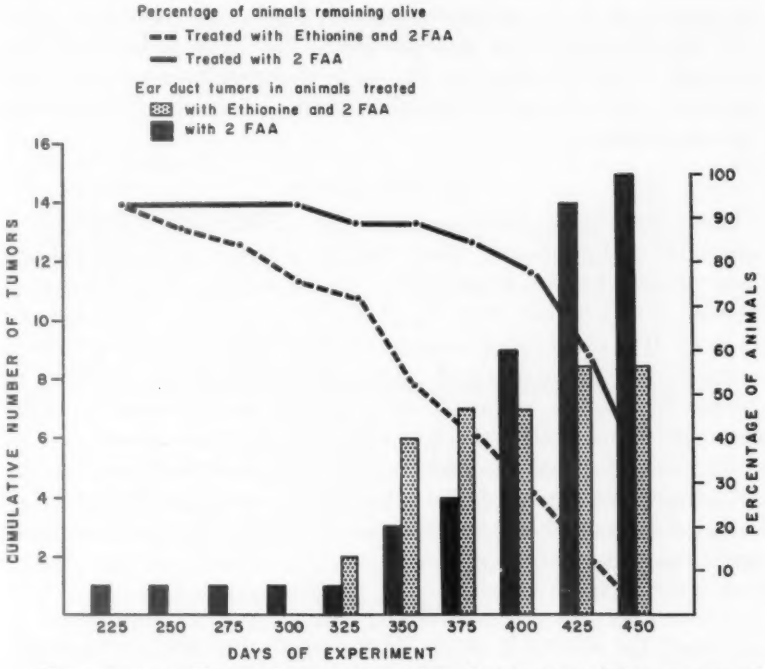
At this time only 40 per cent of the animals in group III remained alive, whereas 85 per cent of the animals in group IV survived. The apparently greater increase in the incidence of tumors in the animals



TEXT-FIGURE 4. The cumulative numbers of mammary gland tumors and the percentage of animals remaining alive in each group.

in group IV in the following 75 days was due partly to the longer period of time and partly to the larger number of animals in which larger quantities of the carcinogen were permitted to act.

By the 450th day, 18 of 25, or 72 per cent, of the animals in group III had 38 grossly apparent tumors. Of the 27 animals in group IV, 15, or 55 per cent, had 22 grossly apparent tumors.



TEXT-FIGURE 5. The cumulative numbers of ear duct tumors and the percentage of animals remaining alive in each group.

Mammary Gland Tumors

Mammary gland tumors developed earlier, grew more rapidly and were more numerous in the animals in group III than in those in group IV (Text-fig. 4). Thus, by the 375th day, 14 of the 25 animals in group III had a total of 25 mammary tumors. However, at this time, only 5 of the 27 animals in group IV had a total of 6 such tumors. At the end of the experiment (450 days) 15 of the animals in group III had a total of 27 mammary tumors. Only 6 of the animals in group IV had developed a total of 7 mammary tumors.

All of the mammary tumors were adenocarcinomas with varying degrees of differentiation. There were varying amounts of infiltration of

adjacent structures. Occasionally metastasis to the lungs had occurred.

Since the majority of the animals in groups I and II were permitted to live until the experiment was terminated, only a limited amount of breast tissue of ethionine-treated animals (group II) was available for comparison with that from a control group (group I) in the early phases of the experiment when the mammary gland tumors began to appear. The mammary ducts of the animals in group II were slightly more cellular than those in the animals in group I. These differences may represent only those occurring naturally among individual animals and thus may not be due to ethionine. The more abundant breast tissues from animals in groups I and II examined later in the experiment had no significant differences.

Ear Duct Tumors

Early in the study, more ear duct tumors developed in the animals in group III. Later, however, partly because a larger number of animals were permitted to live longer, more animals in group IV had a greater number of such tumors (Text-fig. 5). By the 375th day, 6 of the animals in group III had a total of 7 ear duct tumors whereas only 3 animals in group IV had a total of 4 such tumors. By the 450th day, however, 13 animals in group IV had a total of 15 ear duct tumors compared to the 8 animals in group III which had a total of 9 ear duct tumors.

These tumors were all squamous cell carcinomas which arose in the sebaceous gland (Zymbal's glands) about the ear ducts. Infiltration of the skull bones and adjacent tissues occurred frequently. Pulmonary metastasis was occasionally present.

Other Tumors

Two of the animals in group III apparently had single intra-ocular tumors. One animal in group IV had a carcinoma involving and probably arising in the salivary glands.

Multiple Tumors

Multiplicity of tumors occurred with equal frequency, approximately 60 per cent, in the animals of groups III and IV. Table I lists the various combinations observed.

Discussion

Loss of weight, atrophic changes in organs with rapid turn-over of protein materials and retardation of general body growth have been noted during the periods of administration of DL-ethionine. Following cessation of administration, rapid weight gain, rapid regeneration of injured organs, and rapid body growth occur. The effects of DL-ethionine

on ductal epithelium of the mammary glands of the rat have not been investigated. It appears likely, however, that a detailed study would demonstrate slight changes such as those seen in the salivary glands, the gastric mucosa, and the lacrimal glands. It would be natural then for the ductal epithelium to have a period of active growth after cessation

TABLE I

NUMBER OF TUMORS PER ANIMAL

Number of tumors	Number of animals	
	Group III	Group IV
Single tumors	7	7
2 tumors	7	8
Mammary gland only	2	1
Ear duct only	0	2
Ear duct and mammary gland	3	5
Ear duct and eye	2	
3 tumors (all mammary gland)	1	0
4 tumors (all mammary gland)	1	0
5 tumors	2	0
Mammary gland (4) and ear duct (1)	1	
Mammary gland (3) and ear duct(2)	1	

of ethionine administration. Studies with 2FAA indicate that this agent is most effective in producing tumors during such phases of active growth.

The possibility that restriction of food in the animals in group IV may have been responsible for the reduced incidence of mammary gland tumors was also considered. From previous work with ethionine, it was known that animals receiving the drug lost weight despite a fairly good intake of food. Therefore, it was impossible to pair-feed animals during this period and keep the body weights of those animals receiving ethionine comparable to those receiving Ringer's solution. If the body weights may be taken as indicators of the states of nutrition or effective metabolism of ingested food, then the animals in groups III and IV had similar levels of caloric utilization. The differences in incidences of breast tumors cannot be due, therefore, simply to differences in utilization of ingested food. The excellent review of mammary tumors in the rat by Noble and Cutts [3] contains additional discussion and references to this problem.

Ethionine may have interfered with the metabolism of the epithelium in the mammary glands without affecting their growth rate, so that they were more susceptible to the carcinogenic action of 2FAA. At present there are no data to indicate that this did or did not occur.

These observations are of importance since they indicate a method

of producing larger numbers of tumors at earlier ages in experimental animals. The method may also be useful in potentiating the effect of relatively weak carcinogens or tumor viruses. Further investigation of the cellular and metabolic changes may furnish additional basic information regarding tumor induction.

Summary

A study was made of the effect of the administration of DL-ethionine and N-2-fluorenylacetamide in the induction of tumors in female rats. More of the animals treated with ethionine and N-2-fluorenylacetamide developed more mammary and ear duct tumors earlier than did those receiving the carcinogen alone. The mechanism or mechanisms responsible remain unknown. The chief factor is thought to be the alternate periods of slow and rapid growth of the epithelial tissues in ethionine-treated animals.

References

1. Miyaji, H.; Kusaka, Y.; Koyama, K., and Ishida, K. Hepatic changes in rats due to prolonged D.A.B. and ethionine feeding. *Gann*, 1956, **47**, 577–578.
2. Miyaji, H.; Nishi, H.; Watanabe, S.; Koyama, K.; Tamura, K.; Nasu, K.; Kusaka, H., and Ishihama, S. Carcinogenic effects of ethionine on the D.A.B.-carcinogenesis in rats. *Gann*, 1957, **48**, 585–587.
3. Noble, R. L., and Cutts, J. H. Mammary tumors of the rat: a review. *Cancer Res.*, 1959, **19**, 1125–1139.

The technical assistance of Mrs. Dell Rogers is acknowledged.

ELECTRON MICROSCOPIC STUDIES OF MENINGIOMAS

JOHN KEPES, M.D.

From the Department of Pathology and Oncology, University of Kansas Medical Center, Kansas City, Kans.

Electron microscopy in diseases of the nervous system represents a relatively new field in pathology. The first studies of the central nervous system by this means were undertaken 5 to 6 years ago with the description of the submicroscopic structure of the normal brain and spinal cord. Whereas many outstanding papers have been concerned with the electron microscopic findings in inflammatory, degenerative and, particularly, demyelinating disorders of the nervous system, the first important study of brain tumors was published recently by Luse.[1] In this work certain electron microscopic features of a large group of brain tumors, including astrocytomas, oligodendrogliomas, ependymomas, glioblastomas, papillomas of the choroid plexus, meningiomas, neurolemmomas, and neurofibromas, were discussed.

In the present investigation I have concentrated on the submicroscopic features of meningioma. In this connection 3 features were regarded to be of particular interest: (a) the finer structure of the meningothelial tumor cells; (b) the mechanisms involved in the formation of whorls and psammoma bodies; (c) the origin of meningothelial cells. The question also arose whether details revealed by electron microscopy might shed light on the problem of the neuroectodermal versus the mesenchymal origin of meningiomas.

MATERIAL AND METHODS

Fourteen meningiomas removed by surgery were examined. Immediately after removal, the tissue was cut into small blocks with diameters not exceeding 1 mm. Even while cutting, the tissue was submerged in freshly prepared cold 2 per cent osmic acid fixative buffered to pH 7.5 by sodium veronal buffer and preserved by adding 0.147 mg. of sucrose per 3 ml. of fixative. The tissues were then fixed at 4° C. for one hour. This was followed by dehydration with graded concentrations of ethyl alcohol. The absolute alcohol in the series contained 1 per cent phosphotungstic acid. The blocks were infiltrated with methacrylate and embedded in 10/90 methylbutyl methacrylate with added "Luperco (CDB)" catalyst (50 per cent 2,4,-dichlorobenzoyl peroxide with dibutyl phthalate; prepared by the Lucidol Division of Wallace and Tiernan, Inc.) to promote polymerization. Sections cut on Porter-Blum microtomes with glass and diamond knives were mounted on copper grids covered with carbon. Some sections were stained with 2 per cent uranyl nitrate to increase contrast. Finally, the sections were examined in an RCA 3B electron microscope.

This work was supported by United States Public Health Service Grant No. H-4879.
Accepted for publication, June 2, 1961.

Sections for light microscopy were taken from the same tumors. These were fixed in 10 per cent formol, embedded in paraffin and stained with hematoxylin and eosin and by the van Gieson, Masson, and the Gomori trichrome methods, the periodic acid-Schiff (PAS) and elastica stains, and by the Gordon-Sweet reticulin impregnation method. Histologically, most tumors belonged to the meningothelial variety of meningiomas, 2 were of the so-called fibroblastic variety. None of the 14 neoplasms examined appeared to be malignant.

Results and Discussion

The Fine Structure of the Tumor Cells

The meningothelial cells varied in shape from round to elongated forms. Cell membranes were well-defined in most areas. Luse[1] emphasized that the complex plicated interdigitating plasma membranes of these cells caused the indistinct appearance of cell boundaries with light microscopy. We have also encountered such interdigitation, but there were just as many areas where the cell membranes were linear and quite straight. An occasional cell exhibited a straight cell membrane facing one of its neighbors, while having interdigitations with other adjacent cells (Fig. 1). The nuclei were sharply delineated, with a well-defined nuclear membrane. The chromatin in most nuclei was finely granular and evenly distributed, without conspicuous clumping. This was in accordance with the "vesicular" appearance of most meningothelial cells when viewed with light microscopy. Of the intracytoplasmic structures, mitochondria and endoplasmic reticulum displayed no unusual features.

The most characteristic finding consisted of the abundance of fine fibrils in the cytoplasm. These fibrils were of equal thickness and arranged for the most part in parallel bundles with occasional criss-crossing (Figs. 2 and 3). Their density around the nucleus and in the periphery of the cell was about equal. The use of 1 per cent phosphotungstic acid (PTA) in the alcohol series made the fibrils particularly conspicuous. One of Luse's illustrations[1] showed that she too had encountered these fibrils in meningothelial cells.

In studies based on light microscopy there was a certain controversy about the existence of intracellular fibrils in meningothelial cells. Mallory[2] described cytoplasmic fibrillary structures in meningioma tumor cells which he called "fibroglia" and regarded as indicative of the fibroblastic nature of these cells. Bailey,[3] however, claimed that fibroglia fibers were present only in fibroblasts mixed with the real meningothelial cells, and that the latter did not possess such structures.

It is not easy to differentiate between elongated meningothelial cells and fibroblasts. According to Bailey's criteria, however, the central core of a cellular whorl (i.e., the innermost cells) should be accepted as true

meningothelial cells. At the periphery of the whorl, fibroblasts may join the laminated structure.

The present studies, however, showed the existence of these fibers in every cell of the whorls, including the innermost ones and even the central cell if the whorl was built around such a tumor cell rather than a blood vessel or other structure. The existence of fibroglia or "desmofibrils" in meningothelial cells has been debated. On the other hand, they could be impregnated with silver salts by Costero and associates[4,5] in tissue cultures from meningiomas. Romhanyi,[6] using polarizing microscopic methods, expressed a belief in the existence of submicroscopic fibrils in meningioma cells. Electron microscopy thus verifies this indirect assumption based on the appearance of meningiomas in polarized light. With the electron microscope the fibrils were present in every meningioma examined, in the meningothelial as well as the fibroblastic varieties. Of course the fibers impregnated by Costero and co-workers undoubtedly represented greatly condensed elementary fibrils.

The function of these fibrils is not apparent, and one may only speculate on this matter. They probably have a supportive function and also, possibly, a role in the formation of the cellular whorls. Wherever cells in such whorls were examined, the intracytoplasmic fibrils followed the curve and spiral of the cell body. There were also cells which apparently were not participating in whorl formation; the external shape of these was unremarkable, yet inside the cytoplasm a marked whorling of the fibrils could be observed (Fig. 4). A similar arrangement of fibrils was seen in the central cells of whorls.

It appears that this phenomenon is probably the first expression of the whorling tendency in a meningothelial cell. The intracellular alteration, undetected by light microscopy, would be followed later by participation of the whole cell body in the building of a whorl. This finding is in accordance with the view of Kersting and Lennarzt[7] who observed the whorl formation in tissue cultures of meningiomas. They stated that the whorl was initiated by the deformity of a single cell, which became the center of the whorl. Naturally, this view requires modification somewhat when considered *in vivo,* where whorls can develop around blood vessels or collagen fibers. At any rate, I suggest that the "cell deformation" noted by Kersting and Lennarzt begins at the submicroscopic level.

Why does the whorling of the fibrils occur? There are two possible answers to this question. One is that streams and currents develop in the semifluid substance of the cytoplasm and the whorling of the fibrils is simply a visible expression of these changes. The other possibility is that the fibrils themselves have an inherent tendency, possibly due to their

molecular structure, to form whorls and in turn cause the whole cell to participate in whorl formation.

The Mechanism of the Psammoma Body Formation from the Whorls

According to information gained by light microscopy, the whorls are first entirely cellular; later their centers become hyalinized and eventually calcified. The occasional production of spherical proteinaceous bodies (pseudopsammoma bodies) by the meningothelial cells without collagen fibers or a tendency for calcification has been described in another paper.[8] In low power electron micrographs the cellular whorls were easily recognizable, and in some of these (Fig. 5) no evidence of degeneration of the central cell was seen.

As for the whorls with "hyalinized" centers, these centers looked more or less homogeneous under the light microscope, but they were far from homogeneous with electron microscopy. A mixture of elements derived from the cytoplasm (mitochondria with thick membranes and poorly defined cristas, and fragments of cytoplasmic fibrils) were observed, as were collagen fibers of varying thickness, recognized by their characteristic banding. The cytoplasmic membrane of the cells immediately lining this area was more or less fragmented, with a continuity existing between the interior of the cell and extracellular cytoplasmic particles and collagen fibers (Figs. 6 to 8). In some instances, dense amorphous particles were encountered in the centers of the whorls. These were morphologically analogous to structures described by Luse[1] in oligodendrogliomas. She was of the opinion that these represented early foci of calcification in oligodendrogliomas, and the same explanation seems likely in our case—i.e., the particles represented early calcium deposits in psammoma bodies.

As for the origin of the elements in the center of the whorls, the cytoplasmic structures evidently were derived from the neighboring cells. They became extracellular because of cellular degeneration and the breakdown of cell membranes.

The Origin of Collagen Fibers in the Whorls

The presence of collagen fibers in the middle of the whorls raises a more difficult question. Where do collagen fibers in meningiomas come from? It is true that an occasional whorl may develop around a preexisting collagen fiber or, perhaps, a bundle of fibers, but it is a generally accepted notion that initially "pure" cellular whorls eventually develop hyalinized centers, as a process of aging. As has been shown, the hyalinization indicates an admixture of collagen fibers and degenerating cells. These fibers then had to develop within the whorls. Their central location

would certainly relate them to the innermost cell of the whorls, the very cell Bailey [8] and others have recognized as the typical meningothelial cell.

The exact manner of collagen formation is not, as yet, exactly known. Electron microscopic studies by Porter and Pappas [9] in the chick embryo dermis suggested that the formation of collagen fibers was closely related to the cell membranes of fibroblasts. In 3-dimensional pictures produced by shadow casting, a blending of collagen fibers into the ectoplasm of the fibroblasts could be observed. More recently, Yardley, Heaton, Gaines and Shulman [10] described the formation of collagen in tissue culture of chick embryo heart fibroblasts. They found "marginal condensation" or "marginal filaments" in the cytoplasm of fibroblasts which in their opinion were related to the formation of collagen. These active fibroblasts had a loss of definite outer cell membrane. They also described intracytoplasmic fibrils in the fibroblasts and named them "endoplasmic condensation."

The cytoplasmic fibrils found in the meningothelial cells were at least morphologically analogous to the "endoplasmic condensation" of fibroblasts described by Yardley and co-workers.[10] They were also very similar to the intracytoplasmic fibrils described by Hama in mesothelial cells.[11] Marginal fibrils or condensation were not encountered, but in some sections (Fig. 9) the collagen fibers were very closely adjacent to the tumor cells. They ran parallel with the long axis of the cell and the intracytoplasmic fibrils, and appeared to be blending with the cell membrane which was focally indistinct.

In favor of collagen formation by meningothelial cells we can thus list (a) the development of collagen (particularly of young thin fibers) in areas completely surrounded by meningothelial cells, usually in the centers of whorls; and (b) tumor cells with indistinct cell membranes with very closely adjacent collagen fibers. We do not know whether or not the "marginal condensations" described by Yardley and co-workers [10] in tissue cultures are also features of human fibroblastic activity *in vivo*. They were not found in meningothelial cells, and this could be held against an assumption of their fibroblastic activity. Future studies of the formation of collagen in human connective tissue and in fibromas may give a better basis for comparison. Apart from this, the development of collagen in the center of meningothelial whorls suggests the ability of meningothelial cells to produce collagen *in vivo*. Benedek and Juba,[12] Morley,[13] and, in their very elaborate study, Costero, Pomerat, Jackson and Barroso-Moguel [4] noted the fibroblast-like characteristics of meningothelial cells in tissue culture. According to our present findings, these

characteristics can be observed in the tumor cells even without the dedifferentiation that occurs in tissue cultures.

Summary

Fourteen surgically removed meningiomas were examined with the electron microscope. The boundaries between the tumor cells were straight in some areas, interdigitating in others. The fine structure of the tumor cells of the meningothelial variety was not different from cells in "fibroblastic" meningiomas. A common feature of the meningothelial cells was the presence of fine intracytoplasmic fibrils. The whorling of these fibrils was observed in several tumor cells, and it was assumed that this represented the first visible expression of the whorling tendency of these cells.

Whorls with central "hyalinization" were associated with a breakdown of the cytoplasmic membrane in the innermost cells and an admixture of collagen fibers with degenerating cellular elements.

The development of collagen fibers in an area completely surrounded by meningothelial cells indicated the probability that these elements possessed fibroblastic activity. Collagen fibers running parallel and closely adjacent to the cell membrane of the tumor cells supported this notion. Although the cells failed to exhibit all the morphologic criteria of fiber formation observed in tissue cultures of fibroblasts, our findings suggest that meningothelial cells are a specialized variety of connective tissue cells with a collagen-producing propensity.

References

1. Luse, S. A. Electron microscopic studies of brain tumors. *Neurology*, 1960, 10, 881–905.
2. Mallory, F. B. The type cell of the so-called dural endothelioma. *J. Med. Res.*, 1920, 41, 349–364.
3. Bailey, O. T. Histologic sequences in the meningioma, with a consideration of the nature of hyperostosis cranii. *Arch. Path.*, 1940, 30, 42–69.
4. Costero, I.; Pomerat, C. M.; Jackson, I. J., and Barroso-Moguel, R. Tumors of the human nervous system in tissue culture. I. The cultivation and cytology of meningioma cells. *J. Nat. Cancer Inst.*, 1955, 15, 1319–1339.
5. Costero, I.; Pomerat, C. M.; Barroso-Moguel, R., and Chevez, A. Z. Tumors of the human nervous system in tissue culture. II. An analysis of fibroblastic activity in meningiomas. *J. Nat. Cancer Inst.*, 1955, 15, 1341–1365.
6. Romhanyi, G. Histopathologic studies on submicroscopic structure of fibers. *Orvosok lapja*, 1947, 3, 1569–1574.
7. Kersting, G., and Lennarzt, H. *In vitro* cultures of human meningioma tissue. *J. Neuropath. & Exper. Neurol.*, 1957, 16, 507–513.
8. Kepes, J. Observations on the formation of psammoma bodies and pseudopsammoma bodies in meningiomas. *J. Neuropath. & Exper. Neurol.*, 1961, 20, 255–262.

9. PORTER, K. R., and PAPPAS, G. D. Collagen formation by fibroblasts of the chick embryo dermis. *J. Biophys. & Biochem. Cytol.*, 1959, **5**, 153–166.

10. YARDLEY, J. H.; HEATON, M. W.; GAINES, L. M., JR., and SHULMAN, L. E. Collagen formation by fibroblasts: preliminary electron microscopic observations using thin sections of tissue cultures. *Bull. Johns Hopkins Hosp.*, 1960, **106**, 381–393.

11. HAMA, K. The fine structure of the desmosomes in frog mesothelium. *J. Biophys. & Biochem. Cytol.*, 1960, **7**, 575–578.

12. BENEDEK, L., and JUBA, A. Meningeom- und Gliom-Gewebskulturen. *Ztschr. ges. Neurol. u. Psychiat.*, 1942, **174**, 493–503.

13. MORLEY, T. P. The morphology of meningiomas grown in culture. *J. Neuropath. & Exper. Neurol.*, 1958, **17**, 635–643.

I wish to express my thanks to Dr. Peter Rasmussen, Assistant Professor of Pathology, University of Kansas Medical Center, for his valuable help and advice in the field of electron microscopy, and to Dr. William P. Williamson, Professor of Neurosurgery, for kindly supplying the operative specimens.

[*Illustrations follow*]

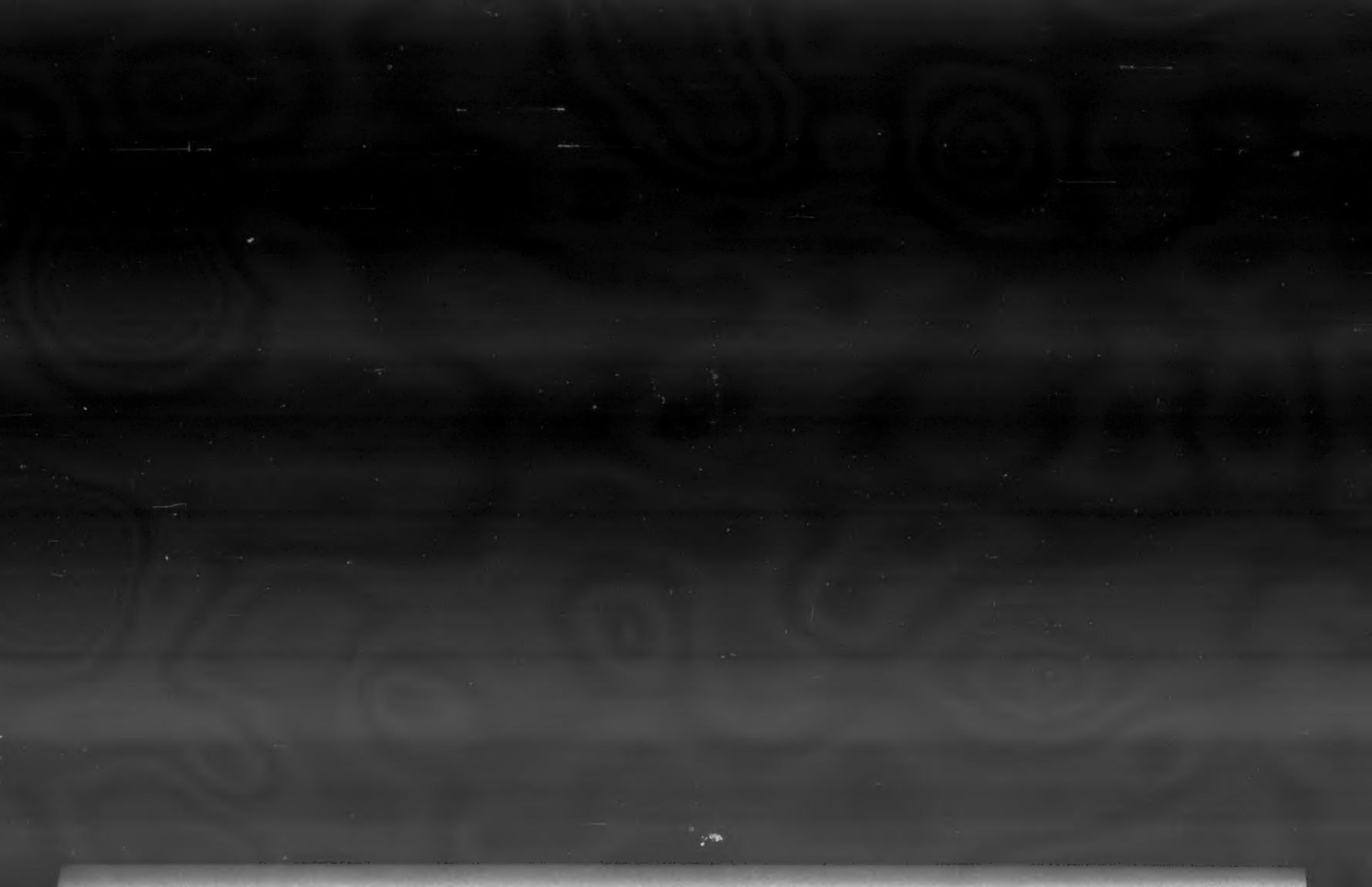
Legends for Figures

Key:

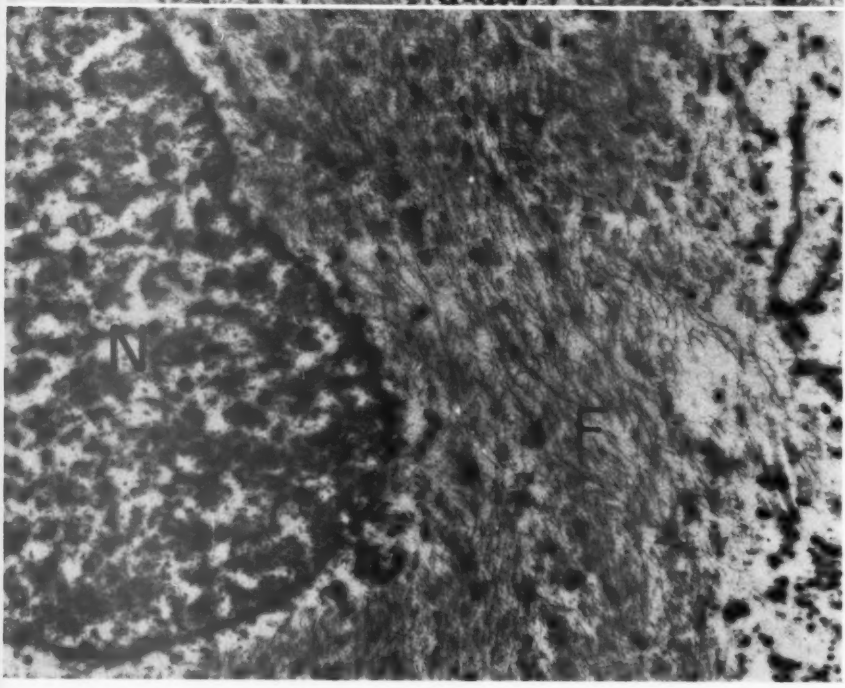
N = nucleus
F = intracellular fibril
CB = cell border
CO = collagen
M = mitochondria
CA = calcium

Fig. 1. Meningothelial meningioma. Cell boundaries are straight in some areas and interdigitating in others. The straight cell borders cause the "epithelioid" appearance with conventional light microscopy; the interdigitating membranes are not readily resolved at such magnification and give the impression of syncytium formation. × 5,400.

Fig. 2. A single meningothelial cell, showing many intracytoplasmic fibrils. Phosphotungstic acid stain. × 17,000.

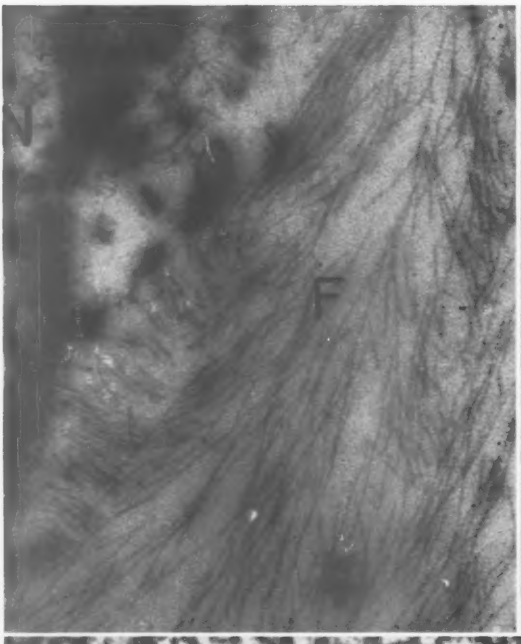


1

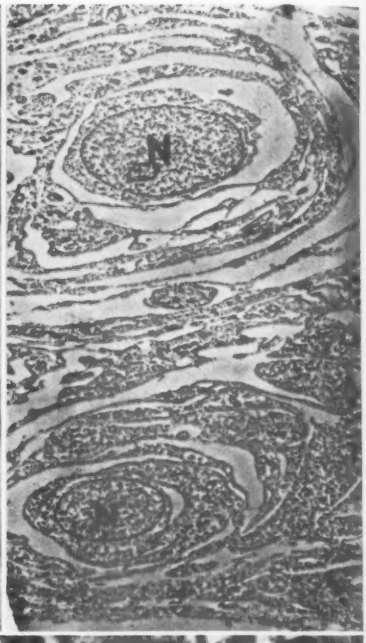


2

3



5



4

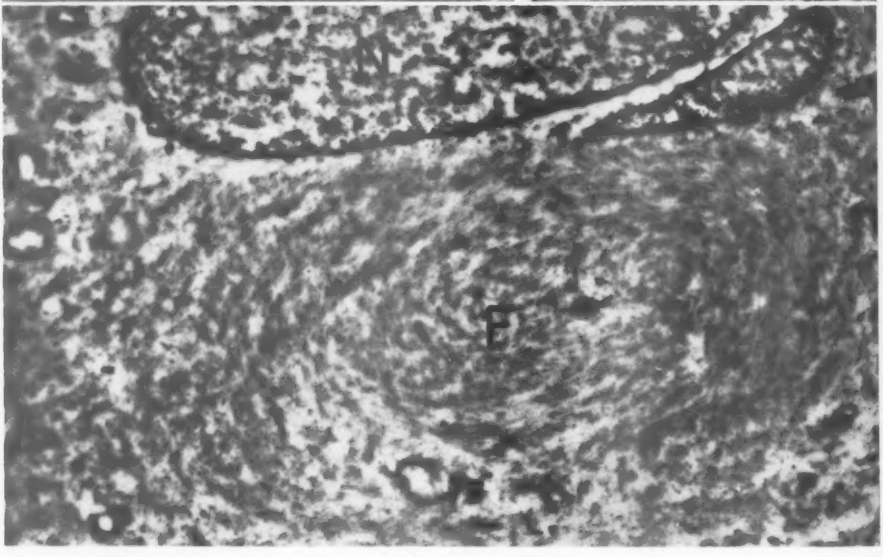
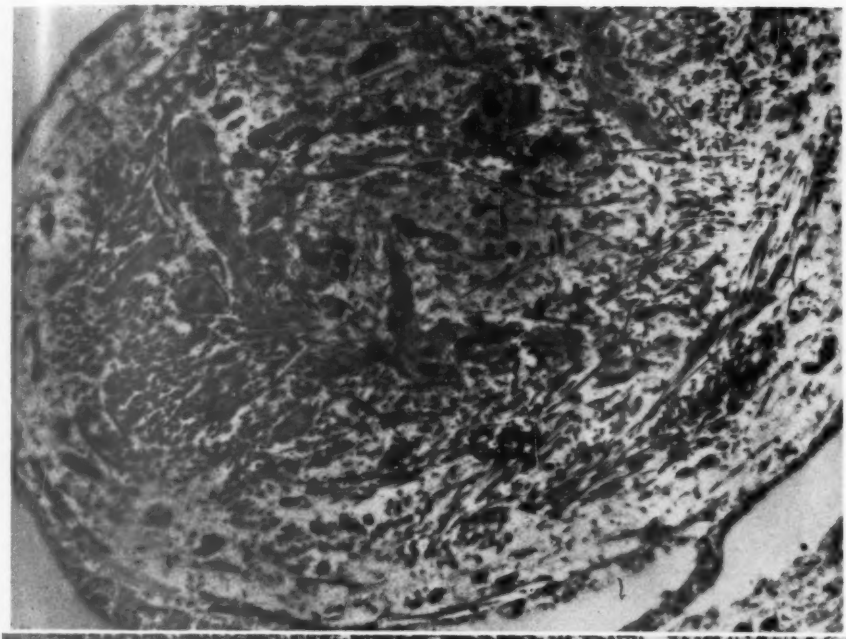


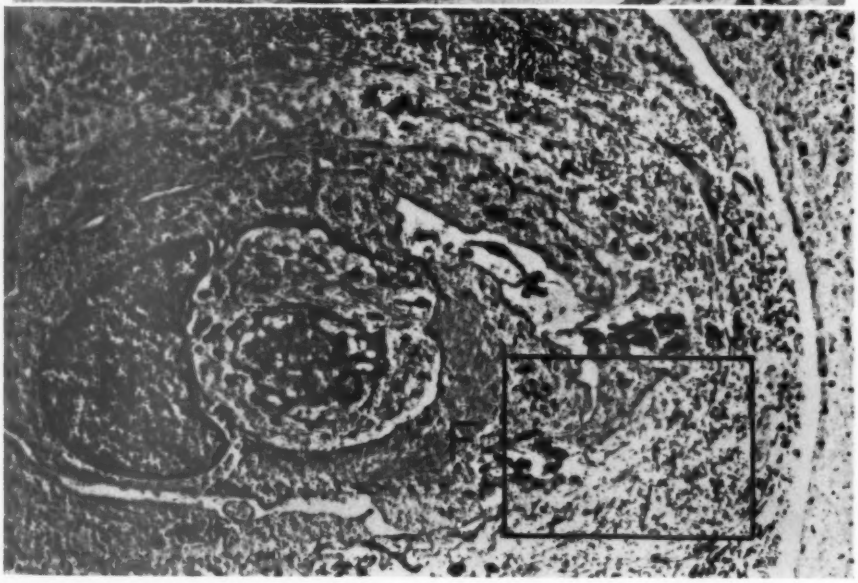
FIG. 3. A higher magnification of the same cell shown in Figure 2. × 65,000.

FIG. 4. The nucleus of this cell is displaced to its periphery. The cytoplasm contains whorling and spiraling fibrils. This arrangement of the fibrils is probably the first visible expression of the whorling tendency of the meningothelial cell. × 15,000.

FIG. 5. A low power electron micrograph of two cellular whorls. No central degeneration is present. × 5,000.



6

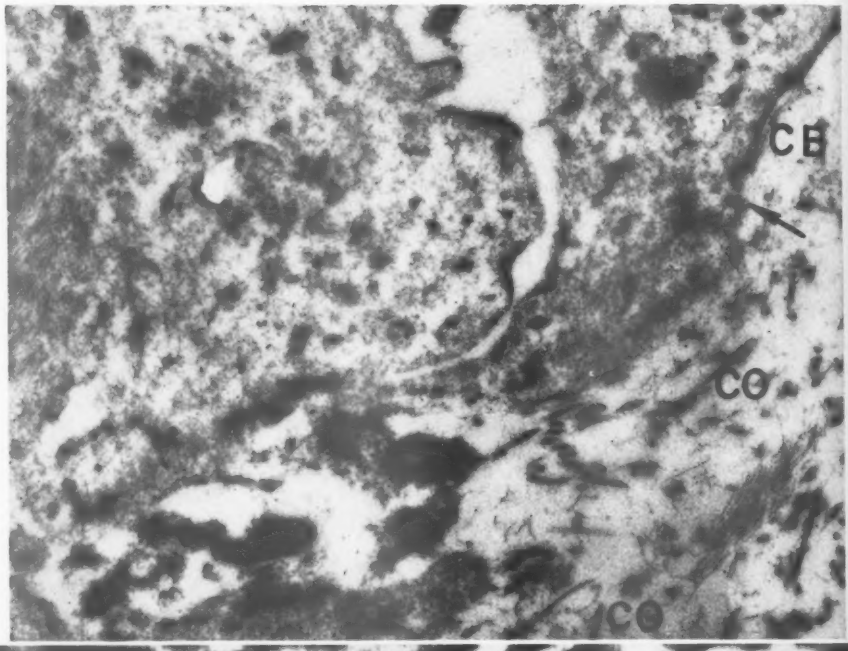


7

FIG. 6. The center of a "hyalinizing" whorl shows a mixture of collagen fibers and structures derived from the cytoplasm, i.e., degenerating mitochondria and bundles of intracytoplasmic fibrils. × 6,000.

FIG. 7. The cells surrounding the center of this whorl show a partial breakdown of their membranes. The center is occupied by amorphous dark clusters, probably early deposits of calcium. × 4,000.

8



9

FIG. 8. Higher magnification of the area shown in Figure 7. There is a mixture of collagen fibers and degenerating cytoplasmic elements. The breakdown of the cell membrane is clearly seen (arrow). × 28,000.

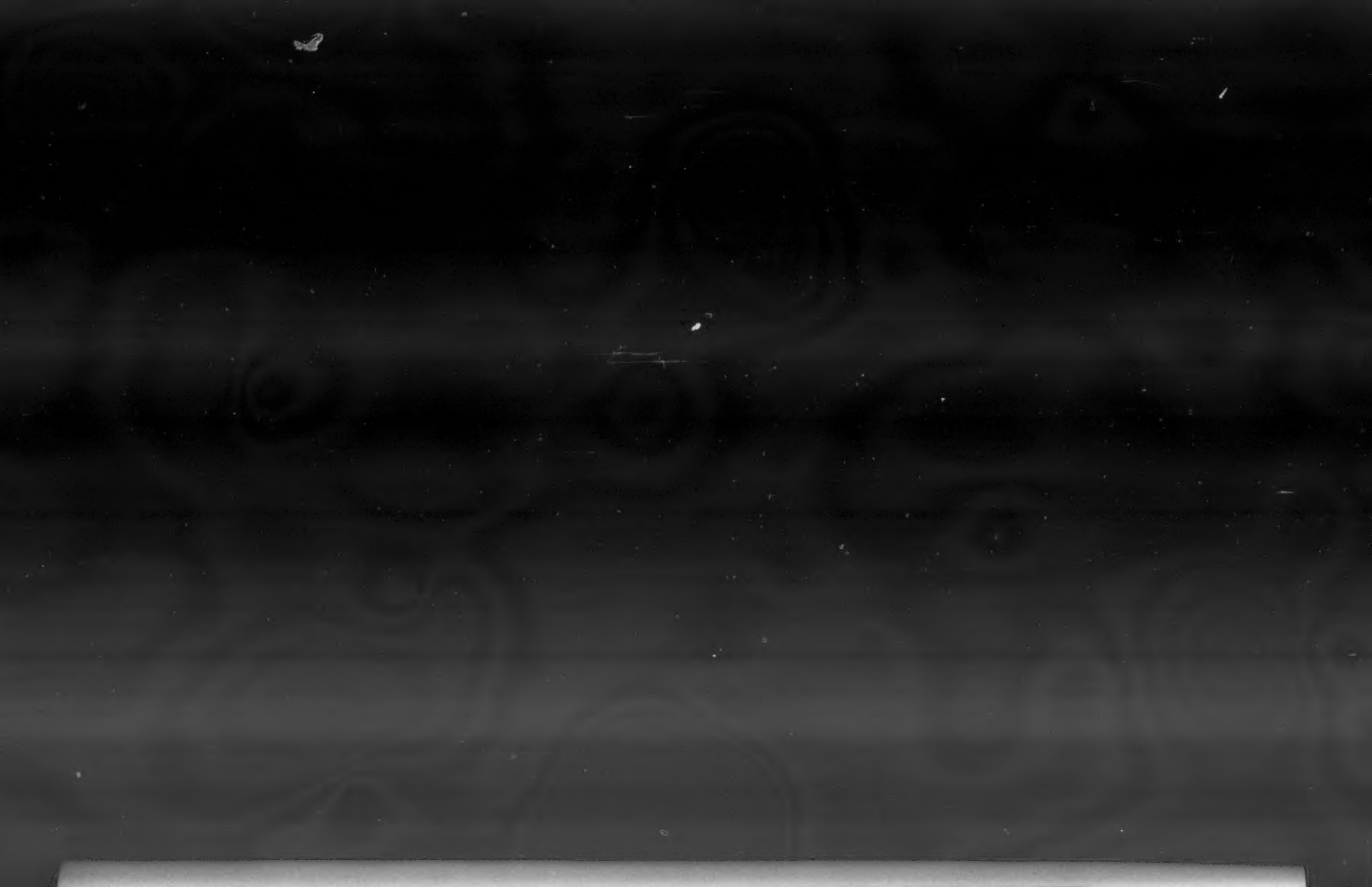



FIG. 9. Collagen fibers are closely adjacent to the cell membrane of this meningothelial tumor cell. The fibers appear to blend with the cell wall which is somewhat indistinct in a few segments (arrows). × 27,000.